Research Article

Comparison of Cardiac miRNA Transcriptomes Induced by Diabetes and Rapamycin Treatment and Identification of a Rapamycin-Associated Cardiac MicroRNA Signature

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Rapamycin (Rap), an inhibitor of mTORC1, reduces obesity and improves lifespan in mice. However, hyperglycemia and lipid disorders are adverse side effects in patients receiving Rap treatment. We previously reported that diabetes induces pansuppression of cardiac cytokines in Zucker obese rats (ZO-C). Rap treatment (750 μg/kg/day for 12 weeks) reduced their obesity and cardiac fibrosis significantly; however, it increased their hyperglycemia and did not improve their cardiac diastolic parameters. Moreover, Rap treatment of healthy Zucker lean rats (ZL-C) induced cardiac fibrosis. Rap-induced changes in ZL-C's cardiac cytokine profile shared similarities with that of diabetes-induced ZO-C. Therefore, we hypothesized that the cardiac microRNA transcriptome induced by diabetes and Rap treatment could share similarities. Here, we compared the cardiac miRNA transcriptome of ZL-C to ZO-C, Rap-treated ZL (ZL-Rap), and ZO (ZO-Rap). We report that 80% of diabetes-induced miRNA transcriptome (40 differentially expressed miRNAs by minimum 1.5-fold in ZO-C versus ZL-C; $p \leq 0.05$) is similar to 47% of Rap-induced miRNA transcriptome in ZL (68 differentially expressed miRNAs by minimum 1.5-fold in ZL-Rap versus ZL-C; $p \leq 0.05$). This remarkable similarity between diabetes-induced and Rap-induced cardiac microRNA transcriptome underscores the role of miRNAs in Rap-induced insulin resistance. We also show that Rap treatment altered the expression of the same 17 miRNAs in ZL and ZO hearts indicating that these 17 miRNAs comprise a unique Rap-induced cardiac miRNA signature. Interestingly, only four miRNAs were significantly differentially expressed between ZO-C and ZO-Rap, indicating that, unlike the nondiabetic heart, Rap did not substantially change the miRNA transcriptome in the diabetic heart. *In silico* analyses showed that (a) mRNA-miRNA interactions exist between differentially expressed cardiac cytokines and miRNAs, (b) human orthologs of rat miRNAs that are strongly correlated with cardiac fibrosis may modulate profibrotic TGF-β signaling, and (c) changes in miRNA transcriptome caused by diabetes or Rap treatment include cardioprotective miRNAs indicating a concurrent activation of an adaptive mechanism to protect the heart in conditions that exacerbate diabetes.

1. Introduction

Obesity and diabetes are metabolic diseases that increase risks for cardiovascular, immune, and inflammatory disease.

Chronic inflammation in patients with obesity and diabetes is characterized by an impaired immune response and increased risk of infections [1, 2]. We recently reported that diabetic Zucker obese (ZO) rats exhibit an intracardiac

cytokine protein expression profile that reflects deficient host defense compared to that of age-matched healthy Zucker lean (ZL) rats [3]. Moreover, we observed that host defense deficiency entailed suppression of both proinflammatory and anti-inflammatory cytokines. We also reported that ZL and diabetic ZO rats exhibited differential metabolic, cardiac structural, functional, and immune responses to rapamycin, an immunosuppressive agent that inhibits the mechanistic target of rapamycin complex 1 (mTORC1) [3].

Rapamycin (Rap) is a macrolide antibiotic and is used as an effective immunosuppressant during solid organ transplantation [4–6]. It is used as an anticancer drug because mTORC1 signaling is hyperactivated in up to 70% of human cancers [7–12]. mTORC1 inhibition has been proposed as an effective strategy for stabilization of atherosclerotic plaques [13]. The mTOR signaling network is implicated in cellular senescence, aging, and lifespan regulation, and rapamycin treatment improves lifespan in different model organisms [14–16]. In brief, Rap and rapalogues (new inhibitors of mTORC1) exert several beneficial effects in the treatment of chronic diseases. On the other hand, accumulating evidence from clinical trials indicates that adverse metabolic side effects of Rap treatment include new onset diabetes and lipid disorders [17–19]. While Rap reduced mortality in healthy mice, paradoxically, long-term rapamycin treatment increased mortality in diabetic mice [20]. We recently reported the suppression of intracardiac expression of GM-CSF, IL-2, IFN-γ, and IL-10, as well as increased decorin and prolactin in diabetic rats and rapamycin-treated nondiabetic rats, indicating a similarity in cardiac cytokine signaling associated with both diabetes and Rap treatment [3]. These observations underscore the need for a better understanding of the molecular regulators that mediate the effects of rapamycin in the diabetic heart.

We have shown previously that when diabetic ZO rats were treated for 12 weeks with a low dose of Rap (750 μg/kg/day delivered via subcutaneous injection), they exhibited significant increase in their fasting glucose levels [3]. While Rap treatment suppressed cardiac fibrosis in ZO rats, it induced cardiac fibrosis in heathy ZL rats, suggesting that mTORC1 inhibition exerts differential effects in diabetic versus healthy animals [3]. Recent studies have identified several microRNAs that mediate the effects of Rap treatment in different cancers [21–26]. MicroRNAs are short (~23 nt) noncoding RNA molecules that function as master regulators of networks of gene expression by virtue of their ability to bind hundreds or thousands of mRNAs [27]. The human genome has over 2000 microRNAs that are predicted to regulate one-third of the genes in the genome [28]. To date, there are no reports that describe how Rap treatment modulates the microRNA expression profiles in healthy and diabetic hearts.

Given the similarities between the intracardiac cytokine expression patterns of Rap-treated and diabetic hearts and that induction of diabetes is one of the main adverse effects of Rap treatment, we hypothesized that there could be significant similarities in the cardiac microRNA transcriptome induced by diabetes and Rap treatment. We further hypothesized that Rap treatment may induce a shift towards increased expression of miRNAs implicated in fibrosis in healthy rat hearts. This hypothesis was tested in the present study using the same four rat models (and corresponding individual rats within those four groups) that we used to characterize how diabetes and Rap treatment modulate intracardiac cytokines. ZL rats (ZL-C) served as baseline controls for miRNA expression in the healthy heart. ZO rats (ZO-C) served as the controls for obesity- and diabetes-induced changes in cardiac miRNA expression. Parallel groups of ZL and ZO rats were treated with Rap for 12 weeks to evaluate the effects of Rap treatment on cardiac miRNA expression in healthy and diabetic hearts, respectively.

We used the GeneChip miRNA 4.0 Array (Thermo Fisher Scientific) to profile miRNA expression in placebo or Rap-treated ZL and ZO rat hearts. Here, we report that changes in intracardiac miRNA transcriptomes induced by rapamycin treatment and diabetes shared significant similarities and provide new insights into mechanisms underlying adverse effects of rapamycin. Differentially expressed miRNAs showed significant correlation with cardiac fibrosis in Rap-treated healthy ZL and diabetic ZO rats. This analysis also uncovered a new, Rap-induced cardiac microRNA signature. Additionally, our *in silico* analysis indicated that human orthologs of rat miRNAs that were highly correlated with cardiac fibrosis in the rats used in this study are involved in modulating the profibrotic TGF-β pathway.

2. Methods

2.1. Rapamycin Treatment of Rats. Rap treatment of 8-week-old ZL and ZO rats was performed as described previously [3]. All animal procedures used in this study were approved by the Harry S. Truman Memorial Veterans Hospital (HSTMVH) Subcommittee for Animal Safety and University of Missouri IACUC before commencing. All animals were cared for in accordance with the guidelines for the care and use of laboratory animals (National Institutes of Health publication 85-23). Briefly, 8-week-old ZO (fa/fa) and lean (ZL) rats (Charles River Laboratories) were maintained on ad libitum food and water and housed singly at the HSTMVH animal housing facility under standard laboratory conditions at room temperature 21–22°C. Animals were entrained to have dark cycle (12 hr: awake time) during the day and light cycle (12 hr: sleep time) during the night so that all interactions with animals matched their awake time. Placebo pellets or rapamycin pellets designed to deliver Rap at a concentration of 750 μg/kg/day for 21 days (from Innovative Research of America Inc., Sarasota, FL) were surgically placed under the skin behind the shoulder blades under brief isoflurane anesthesia, and this procedure was repeated 3 times to achieve a 12-week treatment. ZL and ZO rats that received placebo pellets are referred as ZL-C and ZO-, and those received Rap pellets are referred as ZL-Rap and ZO-Rap, respectively.

2.2. Cardiac miRNA Isolation, Microarray Analysis, and Quantitative Real-Time PCR. Frozen heart tissue from saline- and Rap-treated ZL and ZO rats stored at −80°C was powdered under liquid nitrogen, and miRNA isolation was performed using mirVana miRNA isolation kit (Ambion)

TABLE 1: MicroRNAs differentially expressed in ZO-Rap versus ZL-C and their similarity to microRNAs that were differentially expressed in ZL-Rap versus ZL-C.

MicroRNAs	ZO-Rap/ZL-C	ZL-Rap/ZL-C	Link to diabetes, fibrosis, and/or CVD		
			Diabetes	Fibrosis	CVD
miR-200c/b	4.71/4.099	2.98/4.99	Increased [73, 74]	Suppressed [75]	Increased in familial hypercholesterolaemia [76]
miR-7a-1-3p	4.599	6.844	—	—	—
miR-138-1-3p	4.580	4.238	—		Suppresses cardiac hypertrophy [67, 77]
miR-21	4.533	5.305	Increased [59]	Increased [60]	Reduced in hypertension; cardioprotective [61–63]
miR-26b	4.428	5.147	—	—	Increased in hypertensive patients [78]
miR-434	4.345	9.322	Induces insulin resistance [79]		Increases atrial fibrillation, cardiac damage, heart disease [79–82]
miR-155	4.125	4.55	Increased in T1DM & T2DM [31, 32]	Increased [35]	Increased in cardiac hypertrophy and CHD [33, 34]
miR-30e-3p	4.06	4.80	Suppressed by insulin [45]	—	Suppression associates with cardiac injury [46]
miR-328b-3p	3.734	4.807	—	—	—
miR-505-3p	3.652	4.938	—		Increased in familial hypercholesterolaemia [83]
miR-382	3.407	4.56	Increased [84]	Increased [85]	—
miR-499	3.338	3.86	Induces insulin resistance [79]		Increases atrial fibrillation, cardiac damage, heart disease [79–82]
miR-872-5p	3.255	5.519	—	—	Associated with cardiac oxidative stress and atherosclerosis [86, 87]
miR-217	2.996	2.3409	Increased [88]	Increased [89]	Increased in cardiovascular aging [90]
miR-92b-3p	2.942	3.128	—	—	Suppressed in response to hypoxia [47]
miR-362-3p	2.907	6.316	Suppressed in DM patients [91]	Increased in cardiac fibroblasts [92]	Increased in acute myocardial infarction [93]

Please see Supplemental Table 4 for the exact gene expression levels and p values.

following the manufacturer's protocol and quantified using NanoDrop (Thermo Scientific) as described previously [3]. FlashTag™ Biotin HS RNA Labeling Kit for GeneChip® miRNA Array was used for generating miRNA probes as per manufacturer's instructions using 300 ng of miRNA as input per reaction. Hybridization and scanning of the arrays were performed at the Microarray Core Lab at the University of Colorado, Denver, for a fee. GeneChip miRNA 4.0 Arrays are designed to interrogate all mature miRNA sequences in miRBase release 20. CEL files generated from the scanning of arrays were analyzed using the miRNA microarray data QC analysis as described in the Affymetrix Expression Console Software 1.4 user manual for data normalization. The robust multichip analysis (RMA) + DBAG workflow (Rat), that performs quantile normalization and has a general background correction, was used to generate CHP files. Threshold test showed that all CHIP files were within bounds. Significance of differentially expressed miRNAs between different pairs (ZL-C versus ZO-C, ZL-C versus ZL-Rap, and ZL-C versus ZO-Rap, as well as ZO-C versus ZO-Rap) was determined using unpaired two-tailed t-test. cDNA was generated from the previously isolated miRNA using the miScript II

RT Kit (Qiagen, Valencia, CA). Real-time PCR reactions were performed in triplicate using miScript II SYBR Green PCR Kit and prevalidated Qiagen miScript Primer Assays for miR-21-5p (cat. #MS00013216), miR-144-3p (cat. #MS00021833), miR-155-5p (cat. #MS0001701), miR-101b-3p (cat. #MS00012964), miR-26b-3p (cat. #MS00000140), miR-30e-3p (cat. #MS00013426), and miR-34b-3p (cat. #MS0027468). Reactions were performed using the Bio-Rad IQ5 (Bio-Rad, Hercules, CA) under cycle conditions specified by the manufacturer. The expression levels of target miRNAs relative to endogenous control (RNU6-2; cat. #MS00033740, Qiagen) were quantified by a comparative quantitation cycle method. Relative quantification (RQ) values were obtained by determining ΔCt values followed by determining $\Delta\Delta$Ct values and then RQ values via the equation $2(-\Delta\Delta Ct)$.

2.3. Principal Component Analysis (PCA). RMA + DBAG workflow identified 1218 rat miRNAs. Of the 1218 miRNAs, one-way ANOVA (SAS 9.4, PROC ANOVA) revealed 70 miRNAs that were differentially expressed ($p < 0.05$) by at least 1.5 log2-fold between one or more groups. To identify

similarities in miRNA expression patterns between the groups, PCA was performed on these 70 miRNAs using SAS 9.4 software, PROC PRINCOMP.

2.4. In Silico Analysis for Identifying Differentially Expressed Cytokines Targeted by Differentially Expressed miRNAs. NCBI gene database was used to retrieve complete mRNA sequence data for differentially expressed cytokines identified from pairwise comparison of ZO-C or ZO-Rap versus ZL-C that we reported previously [3]. RegRNA software [29] was used to retrieve predicted miRNA binding sites for each of the cytokines. miRNA binding sites for each cytokine mRNA in the differentially expressed cytokine list for a given pairwise comparison (e.g., ZO-C versus ZL-C) were compared with the list of differentially expressed miRNAs in the same pairwise comparison. Then, the data were compiled, and the list of different cytokines targeted by a given miRNA was organized and presented in Table 1.

2.5. In Silico Analysis to Determine Correlation of Fibrosis with Differentially Expressed miRNAs. Human orthologs for miRNAs demonstrating a significant relationship ($p < 0.05$) with cardiac fibrosis were entered into DIANA-miRPath v3.0 software [30] to determine associated pathways in humans, using both Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology-Biological Processes (GO-bp) analyses, and target genes associated with those pathways. Secondary KEGG pathway enrichment analyses and subsequent identification of target genes along significantly enriched pathways were performed using miRNet (http://www.miRNet.ca/).

2.6. Statistical Analysis. Results are reported as means ± SE. Statistical analysis was performed using SigmaStat or SAS 9.4 software. Unpaired two-tailed t-test was performed for pairwise comparisons. A p value < 0.05 was deemed significant. Spearman correlation coefficients were obtained for miRNA expression and measures of cardiac fibrosis, independent of the treatment group, using PROC REG (SAS 9.4).

3. Results

One-way ANOVA of the 1218 rat miRNAs used as probes in this study showed that 70 miRNAs exhibited statistically significant ($p < 0.05$) differential expression by at least 1.5 log2-fold between one or more groups (Figure 1). Principal component analysis (PCA) of these 70 miRNAs showed that ZL-C and ZL-Rap were distinct groups whereas ZO-C and ZO-Rap groups clustered together. This observation suggested that while Rap treatment had strong effects on the miRNA transcriptome of ZL rats, Rap did not alter the miRNA transcriptome of ZO-rats to the same extent.

3.1. Comparison of Cardiac miRNA Transcriptome in ZL-C and ZO-C. We previously reported metabolic and cardiac parameters of ZL-C and ZO-C used in this study and differences in their intracardiac cytokine profiles [3]. In this study, miRNA transcriptomes of the same heart tissues were analyzed. Diabetes induced significant differential expression of a total of 177 cardiac microRNAs in ZO-C (Supplemental
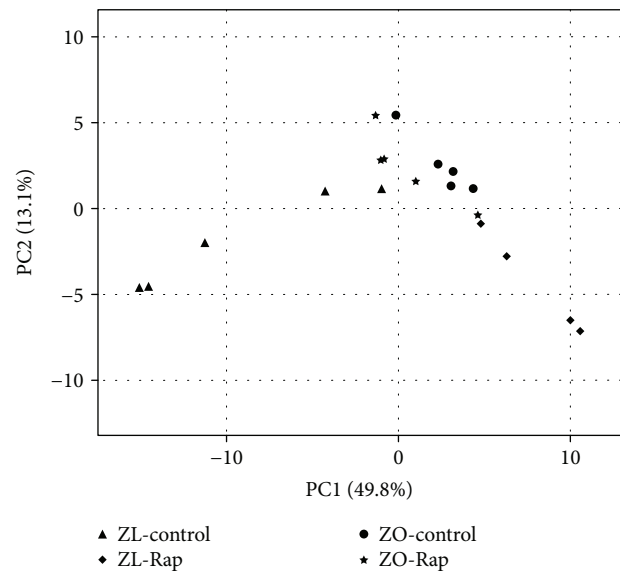

FIGURE 1: PCA analysis of the 70 miRNAs determined to be differentially expressed ($p < 0.05$) by at least 1.5 log2-fold between one or more groups. Comparisons revealed three moderately distinct clusters (1) ZL-control (ZL-C), (2) ZL-Rap, and (3) ZO-control (ZO-C) and ZO-Rap.

Table 1). Specifically, 105 miRNAs showed an increase, and 72 miRNAs showed a reduction in their expression in ZO-C compared to ZL-C. Among them, 40 microRNAs showed differential expression ≥ 1.5 log2-fold (Figure 2). A literature search provided evidence that 20 of these miRNAs are associated with diabetes and/or cardiac fibrosis and/or different cardiovascular diseases (Table 2). The microRNA miR-155-5p, a biomarker that is increased in the plasma of patients with type 1 diabetes [31, 32] and in gingival crevicular fluid of patients with periodontitis and type 2 diabetes [33] and contributes to cardiac hypertrophy and coronary heart disease [34, 35], was increased by 5.21-fold in the ZO rat heart, consistent with the cardiovascular detrimental effects of diabetes. Other miRNAs that have defined roles in cardiovascular damage included miR-872-5p, miR-350, miR-362-3p, miR-223-3p, miR-204, miR-98, miR-217, miR-379, and miR-181-3p (Table 2 and references therein). MicroRNAs that contribute to fibrosis such as miR-21, miR-382, miR-155, miR-223-3p, and miR-217 were also increased in ZO-C hearts compared to ZL-C hearts (Table 2). However, miRNAs that suppress fibrosis (miR-200b/c, miR-411-5p, miR-140, miR-322, and miR-98) and render cardiovascular protective effects (miR-21 and miR-140) were also simultaneously increased in the ZO-C heart indicating the activation of an adaptive mechanism to regulate fibrosis induced by high glucose and insulin resistance.

3.2. Comparison of Cardiac miRNA Transcriptome in ZL-C and ZL-Rap. A total of 221 cardiac miRNAs were differentially expressed between ZL-C and ZL-Rap that showed statistical significance. Out of these, 131 cardiac miRNAs had increased expression and 90 miRNAs had decreased expression in ZL-Rap compared to ZL-C (Supplemental Table 2).
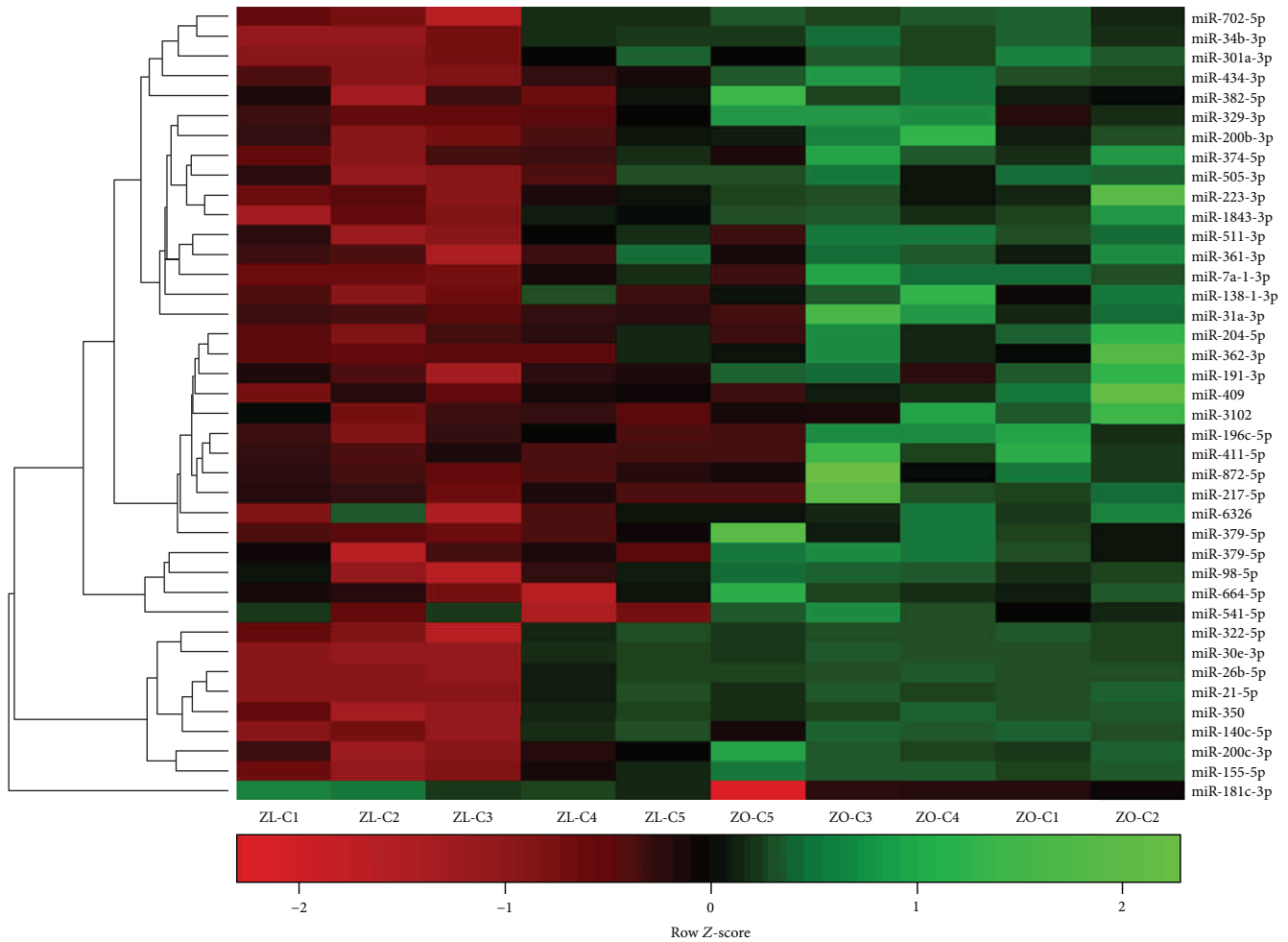
FIGURE 2: Hierarchal cluster heat map of differentially expressed cardiac miRNAs in ZL-C ($n = 5$) vs. ZL-Rap ($n = 4$) that exhibited a 2.25-fold change in expression in either direction. Red signals indicate lower expression levels, and green signals indicate higher expression levels.

Further selection of miRs that exhibited at least a 1.5 log2-fold difference showed that 68 miRNAs were differentially expressed between ZL-C and ZL-Rap (Figure 3). We found that 32 of these miRNAs (47%) were the same and showed the same directionality of expression as those that were found to be differentially expressed between ZL-C and ZO-C (Figure 4). This accounted for 80% of the differentially expressed cardiac miRNAs in ZO-C. Therefore, there is a remarkable similarity between the cardiac transcriptome activated by Rap treatment in healthy hearts and that activated in response to diabetes. Table 2 lists 20 of these miRNAs that showed the same expression pattern in ZL-Rap and ZO-C and that are also implicated in diabetes and/or fibrosis and/or cardiovascular damage.

Table 3 lists those miRNAs that were significantly differentially expressed by at least 1.5 log2-fold in either direction in ZL-Rap compared to ZL-C and show some relationship with diabetes and/or fibrosis and/or cardiovascular damage according to literature. These miRNAs were not differentially expressed by 1.5 log2-fold in ZO-C compared to ZL-C and therefore were unique to the ZL-Rap hearts. Several miRNAs that are diabetes markers and contribute to insulin resistance,

pancreatic beta cell death, and progression of diabetes are included in this list. For example, miR-29 family miRNAs are significantly increased in patients with T1DM and T2DM and involved in pancreatic beta cell death and progression of diabetes [36–38]. MicroRNAs miR-19a, miR-499, miR-539, miR-363, miR-495, miR-7a, and miR-429, which are reported to be increased in diabetic patients or animal models and/or contribute to insulin resistance, were increased in ZL-Rap hearts suggesting that Rap treatment induces a diabetes-associated miRNA transcriptome in ZL-Rap hearts. However, miR-455 and miR-451, which are reported to be suppressed in diabetes, were found to be increased in ZL-Rap hearts, whereas miR-144 that is shown to be increased in diabetes was suppressed in ZL-Rap hearts [39–41]. Additionally, a miRNA implicated in promoting diabetic wound healing, miR-335, was also among the miR-NAs that had increased transcription in ZL-Rap hearts [42].

Several of these miRNAs are also linked to CVD (Table 3). miRNAs that are associated with exacerbating different CVDs according to literature and increased in ZL-Rap hearts include miR-451, miR29 family, miR-19a, miR-499, miR-539, miR-363, miR-495, and miR-429 (Table 3 and references

TABLE 2: Similarities between differentially expressed microRNAs that modulate diabetes, cardiac fibrosis, and other cardiovascular diseases in ZO-C versus ZL-C and ZL-Rap versus ZL-C.

MicroRNA	ZO-C/ZL-C	ZL Rap/ZL-C	Nature of association with human or animal model pathology		
			Diabetes	Fibrosis	CVD
miR-155	5.21	4.55	Increased in T1DM & T2DM [31, 32]	Increased [35]	Increased in cardiac hypertrophy and CHD [33, 34]
miR-200b/c	5.17/4.00	4.99/2.98	Increased [73, 74]	Suppressed [75]	Increased in familial hypercholesterolaemia [76]
miR-21	4.59	5.28	Increased [59]	Increased [60]	Reduced in hypertension; cardioprotective [61–63]
miR-26b-5p	4.41	5.13	—	—	Increased in hypertensive patients [78]
miR-872-5p	4.32	5.52	—	—	Associated with cardiac oxidative stress and atherosclerosis [86, 87]
miR-411-5p	3.89	7.16	—	Suppresses fibrosis [94]	Increased in abdominal aortic aneurism [95]
miR-382	3.84	4.56	Increased [84]	Increased [85]	—
miR-301a	3.81	4.86	Increased [96]		Increased in the diabetic heart [96]
miR-329	3.81	5.50	—	—	Increased in ischemia [97]
miR-350	3.68	3.61	—	—	Induces pathological cardiac hypertrophy [98]
miR-505-3p	3.66	4.92	—	—	Increased in familial hypercholesterolaemia [83]
miR-140	3.58	4.00	Reduced in platelets [99]	Suppresses fibrosis [100]	Suppresses pulmonary arterial hypertension [101]
miR-322	3.56	3.84	Suppressed by high glucose [102]	Suppresses fibrosis [103]	Improved cardiac function [103]
miR-362-3p	3.48	6.28	Increased [104]	—	Associated with atherosclerosis [105]
miR-374	3.34	5.94	—	—	Increased in cardiac hypertrophy and aneurism [106, 107]
miR-223-3p	3.27	3.56	Suppressed in DM patients [91]	Increased in cardiac fibroblasts [92]	Increased in acute myocardial infarction [93]
miR-204	3.20	7.01	Suppresses insulin [108]	—	Increases endoplasmic reticulum stress [109]
miR-98	3.20	3.41	Increased [110]	Reduces collagen [111]	Reduced in myocarditis [112]; increased in postheart transplant [113]
miR-34b	3.10	3.29	—	—	Reduced in diabetic ischemic heart failure [114]
miR-217	2.99	2.98	Increased [88]	Increased [89]	Increased in cardiovascular aging [90]
miR-541	2.97	3.18	Involved in pancreas development [115]	—	Suppresses cardiac hypertrophy [116]
miR-379	2.85	2.91	—	—	Apoptosis of VSMC [117]

Please see Supplemental Tables 1 and 2 for the exact gene expression levels and p values.

therein). Additionally, loss of miR-144 and miR-542 also exacerbated CVD. Conversely, miR-101b and miR-7a, which are cardioprotective (Table 3 and references therein), are also among the cardiac miRNAs that are increased by Rap treatment.

3.3. Comparison of Cardiac miRNA Transcriptome in ZO-C and ZO-Rap. Rap treatment of ZO-C resulted in differential myocardial expression of 128 miRNAs that included 84 miRNAs with increased expression and 44 miRNAs with suppressed expression (Supplemental Table 3). However, only four miRNAs from this group met the ±1.5 log2-fold change threshold (Figure 5). Associations between these miRNAs to specific diseases are shown in Table 4. miR-743a-5p that mediates mitochondrial oxidative stress was increased in the ZO-Rap heart, but not in the ZL-Rap heart, indicating that this is a unique effect caused by the combination of diabetes and Rap treatment (Table 4) [43]. miR-511-3p was the only miRNA that showed similar expression changes in Rap-treated ZL and ZO rats (suppressed in both cases), and loss of miR-511-3p is associated with minimally oxidized low-density lipid-associated increase in atherosclerosis (Table 4) [44]. There is very little information regarding miR-1843-3p and miR-409b in the literature; however, Rap treatment had opposing effects on the expression of these miRNAs in healthy ZL-C and diabetic ZO-C (Table 4).
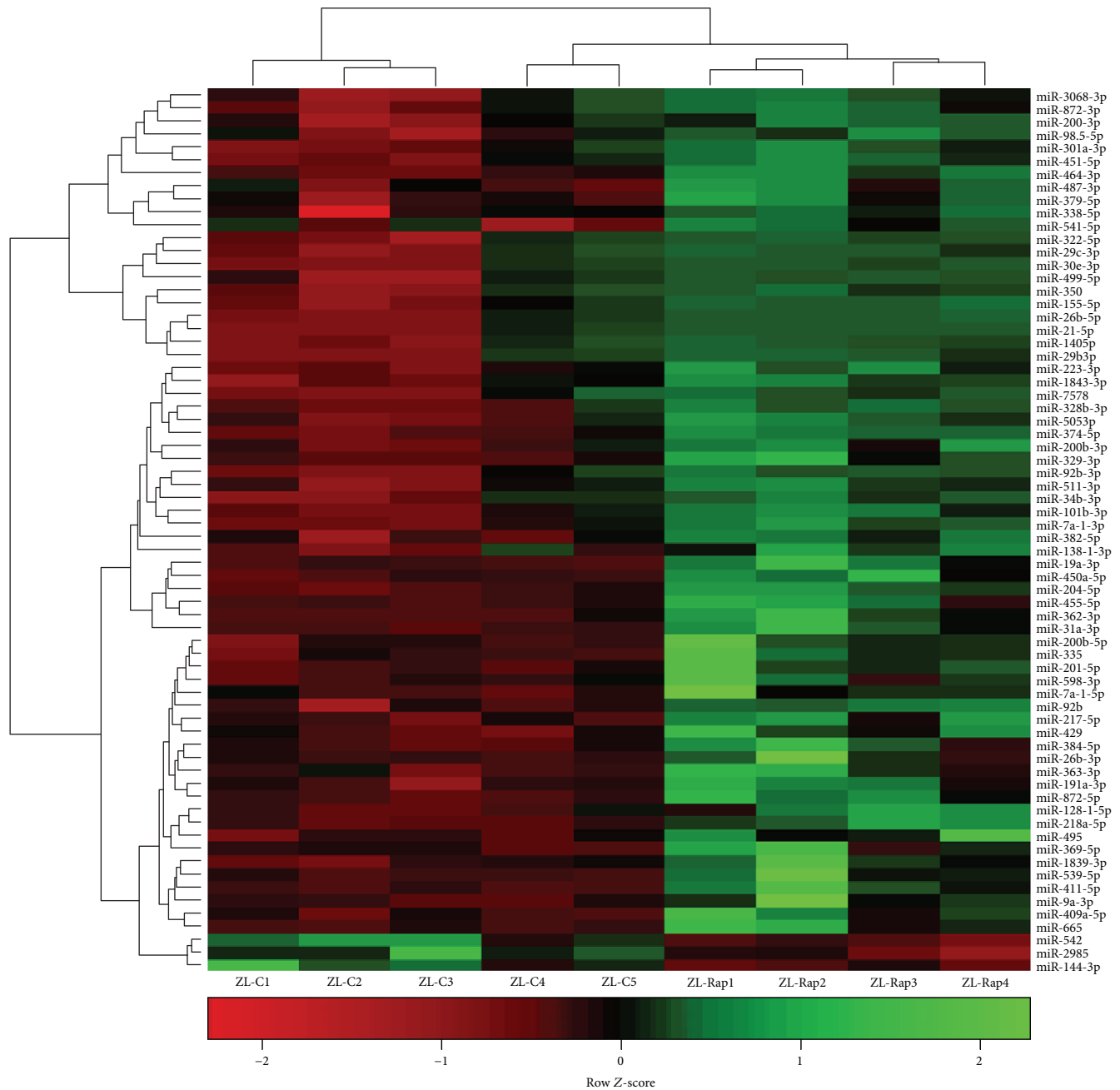
FIGURE 3: Hierarchal cluster heat map of differentially expressed cardiac miRNAs in ZL-C ($n = 5$) vs. ZO-C ($n = 5$) that exhibited a 2.25-fold change in expression in either direction. Red signals indicate lower expression levels, and green signals indicate higher expression levels.

3.4. Comparison of Cardiac miRNA Transcriptome in ZL-C and ZO-Rap. Next, we compared the cardiac miRNA transcriptome of ZO-Rap with that of ZL-C. Of the 116 miRNAs that were differentially expressed between these two groups (Supplemental Table 4), 83 had increased expression and 33 exhibited suppressed expression in ZO-Rap hearts. However, only 27 of these miRNAs met the ±1.5 log2-fold change threshold (Figure 6). Importantly, 17 of these miRNAs were the same as those that were increased in ZL-Rap (Table 1). Among these miRNAs, miR-30e-3p

is shown to be suppressed by insulin [45]; however, another study showed that its suppression is associated with myocardial injury induced by coronary microembolization via autophagy activation [46]. Moreover, miR-92b-3p is shown to be suppressed by hypoxia [47], but its expression is elevated in the heart tissues of both Rap-treated groups. One miRNA in this group, miR-7a-1-3p, is not known to associate with diabetes, fibrosis, or CVD. The remaining miRNAs in this group are associated with either diabetes, fibrosis, or cardiovascular diseases as shown in Table 1.

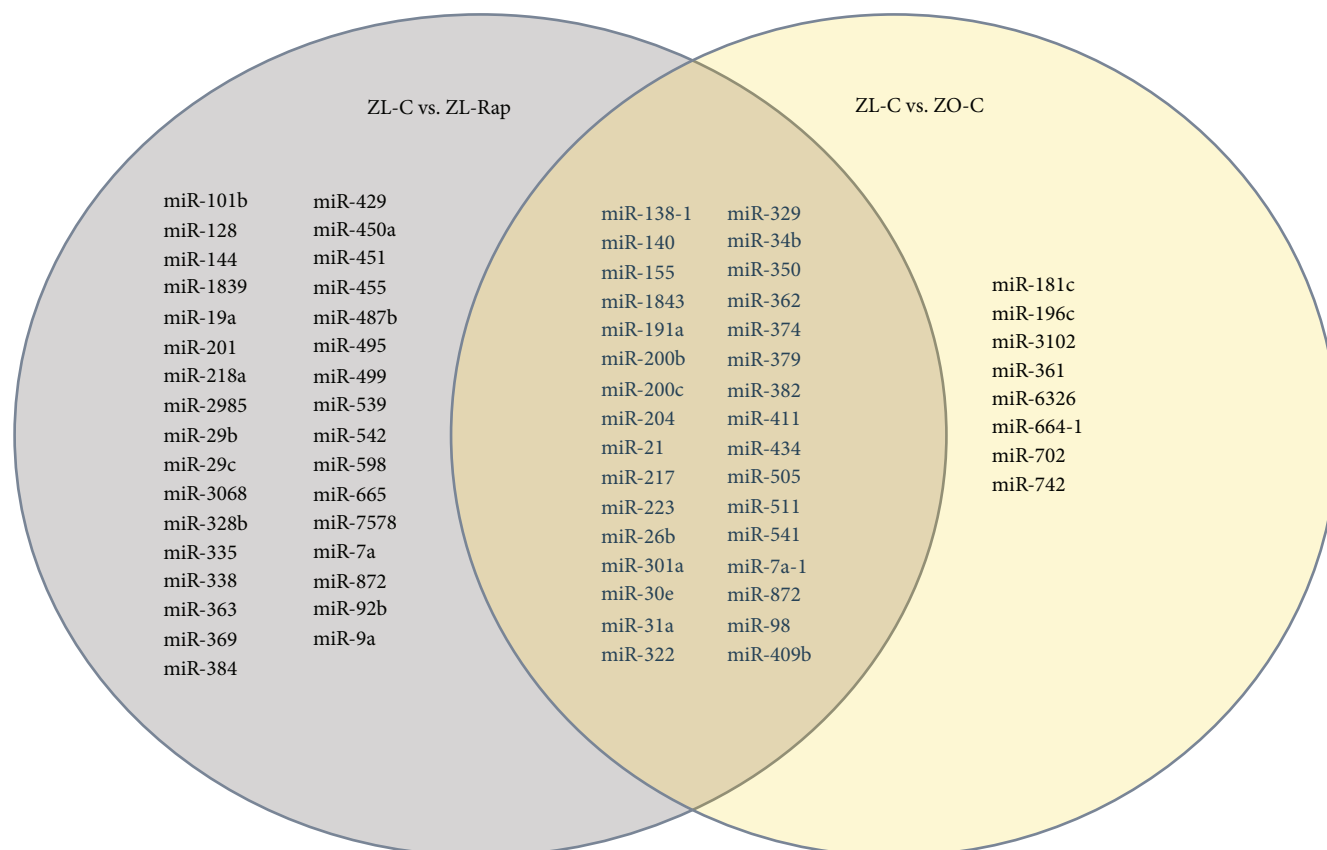| ZL-C vs. ZL-Rap		ZL-C vs. ZO-C	
miR-101b	miR-429	miR-138-1 miR-329	miR-181c
miR-128	miR-450a	miR-140 miR-34b	miR-196c
miR-144	miR-451	miR-155 miR-350	miR-3102
miR-1839	miR-455	miR-1843 miR-362	miR-361
miR-19a	miR-487b	miR-191a miR-374	miR-6326
miR-201	miR-495	miR-200b miR-379	miR-664-1
miR-218a	miR-499	miR-200c miR-382	miR-702
miR-2985	miR-539	miR-204 miR-411	miR-742
miR-29b	miR-542	miR-21 miR-434	
miR-29c	miR-598	miR-217 miR-505	
miR-3068	miR-665	miR-223 miR-511	
miR-328b	miR-7578	miR-26b miR-541	
miR-335	miR-7a	miR-301a miR-7a-1	
miR-338	miR-872	miR-30e miR-872	
miR-363	miR-92b	miR-31a miR-98	
miR-369	miR-9a	miR-322 miR-409b	
miR-384			

FIGURE 4: Venn diagram demonstrating miRNA that exhibited a 2.25-fold change in expression in either direction between ZL-C vs. ZL-Rap (left) and ZL-C vs. ZO-C (right), as well as those that were differentially expressed in both comparisons (center).

TABLE 3: MicroRNAs that modulate diabetes, cardiac fibrosis and other cardiovascular diseases and differentially expressed in ZL-Rap versus ZL-C.

MicroRNAs	ZL-Rap/ZL-C	Link to diabetes and/or CVD	
		Diabetes	CVD
miR-455	6.76	Suppressed [39]	—
miR-451	5.1076	Suppressed [40]	Induces cardiac hypertrophy [40]
miR-101b	5.0625	—	Suppresses cardiac hypertrophy [67, 77]
miR-29b/c	4.75/3.83	Increased in diabetes [36–38]	Correlates with CVD progression in diabetes [36–38, 118]
miR-19a	4.7089	Increased in diabetes [119]	Induces heart failure and vascular inflammation [120–122]
miR-499	3.8636	Induces insulin resistance [79]	Increases atrial fibrillation, cardiac damage, heart disease [79–82]
miR-539	3.7249	Increased in diabetes [123]	Induces mitochondrial fission, cardiomyocyte apoptosis [123, 124]
miR-363	3.7249	Urinary marker for diabetes [125]	Inhibition protects cardiomyocytes from apoptosis [126]
miR-335	3.2761	Improves diabetic wound healing [42]	—
miR-92b	3.0976	—	Increased in heart failure patients [127]
miR-495	3.0976	Urinary marker for diabetes [125]	Involved in causing hypertrophy [128, 129]
miR-7a	2.8224	Inhibits glucose-stimulated insulin secretion [130]	Protects against cardiomyocyte injury [131, 132]
miR-429	2.56	Impairs intestinal barrier in diabetic mice [133]	Causes cardiomyocyte apoptosis [134]
miR-487b	2.3409	—	Mitigates chronic heart failure [135]
miR-144	−2.28	Increased in diabetes [41]	Loss of miR-144 impairs cardioprotection [136]
miR-542	−2.28	—	Involved in aortic calcification [137]

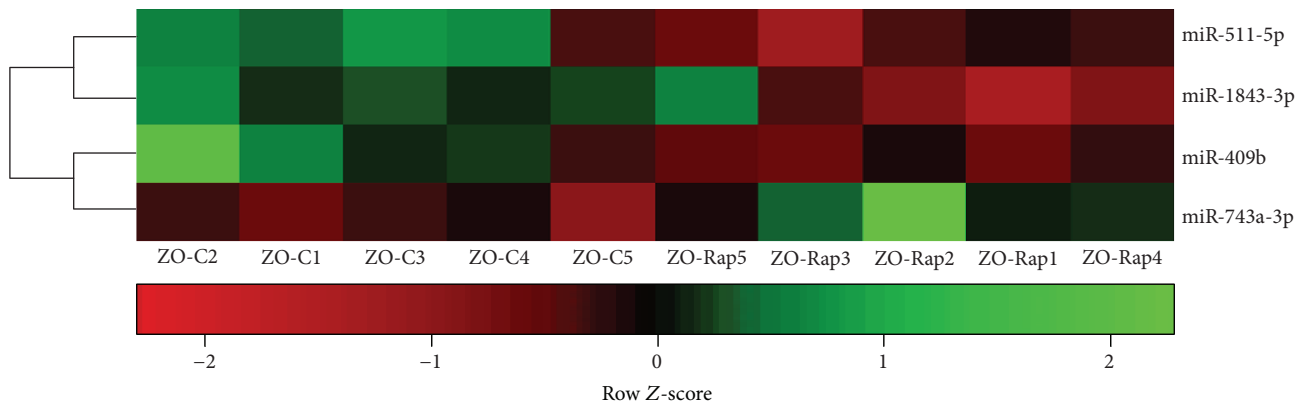Please see Supplemental Table 2 for the exact gene expression levels and p values.

FIGURE 5: Hierarchal cluster heat map of differentially expressed cardiac miRNAs in ZO-C ($n = 5$) vs. ZO-Rap ($n = 5$) that exhibited a 2.25-fold change in expression in either direction. Red signals indicate lower expression levels, and green signals indicate higher expression levels.

TABLE 4: MicroRNAs differentially expressed in ZO-Rap versus ZO-C and their similarity to microRNAs that were differentially expressed in ZL-Rap versus ZL-C.

| MicroRNA | ZO-Rap/ZO-C | ZL-Rap/ZL-C | Nature of Association with human or animal model pathology | | |
			Diabetes	Cardiac fibrosis	CVD
miR-743a-5p	2.25	1.09; p = N.S.	—	—	Mediates mitochondrial oxidative stress [43]
miR-1843-3p	−2.25	2	—	—	—
miR-511-3p	−2.25	−1.7	—	—	Suppressed in moX-LDL-induced VSMC transformation in atherosclerosis [44]
miR-409b	−2.25	1.45; p = N.S.	—	—	—

N.S.: not significant. Please see Supplemental Table 3 for the exact gene expression levels and p values.

Because myocardial expression of these 17 miRNAs is increased by Rap treatment in both ZL and ZO rats, we propose that these miRNAs constitute a Rap-induced cardiac microRNA signature.

3.5. Cytokines Targeted by the Differentially Expressed miRNAs.

We previously reported which intracardiac cytokines were differentially expressed in response to diabetes (ZL-C versus ZO-C) and Rap treatment (ZL-C versus ZL-Rap) [3]. Because there was significant overlap between the miRNAs that were differentially expressed between these two groups, we hypothesized that these miRNAs would play a role in regulating differentially expressed cytokines in these groups. To determine if the differentially expressed miRNA transcriptome exerts the regulation of the corresponding cytokine profile, we performed an *in silico* analysis using miRbase miRNA target analysis. The miRNAs that had binding sites on the 3′ untranslated sites of the mRNAs expressing differentially expressed cytokines from each pairwise comparison were selected. Table 5 shows miRNAs that exhibited increased expression and their target cytokines that carried binding sites for the corresponding miRNAs on the 3′ untranslated regions of their mRNAs. As shown in Table 5, differentially expressed miRNAs had binding sites on the 3′ untranslated regions of most of the differentially expressed cytokines. Therefore, changes in expression patterns of intracardiac cytokines in response to diabetes and rapamycin treatments in rat hearts were associated with changes in their miRNA transcriptomes.

3.6. Differentially Expressed miRNAs That Correlated with Fibrosis and Validated by Real-Time PCR.

We have previously published that ZL rats treated with rapamycin have increased cardiac fibrosis compared with untreated ZL rats [3]. However, in ZO rats, treatment with rapamycin attenuated cardiac fibrosis [3]. In order to determine which specific miRNA may be contributing to cardiac fibrosis, we performed a correlation analysis using all differentially expressed miRNAs that exhibited at least 1.5 log2-fold change in pairwise comparisons. Analysis of the combined data from all treatment groups showed that seven miRNAs were positively correlated (miR-140-5p, miR-155-5p, miR-21-5p, miR-26b-5p, miR-30e-3p, miR-34b-3p, and miR-379-5p; $p < 0.05$) and one miRNA, miR-144-3p, was inversely correlated (miR-144; $p < 0.05$) with the degree of cardiac fibrosis (Figure 7). Of the seven positively correlated miRNAs, there is evidence supporting strong profibrotic activity for miR-21 and miR-34b [48–52]. In contrast to the positive correlation with fibrosis observed here, miR-140 is considered antifibrotic [53, 54]. miR-155 and miR-34b demonstrate both pro- and antifibrotic activities [55, 56]. miR-140, the only one demonstrating an inverse association, has well-documented antifibrotic activity [57, 58]. To further validate differential expression of some of these miRNAs, we performed quantitative RT-PCR. As shown in Figure 7, miRNAs
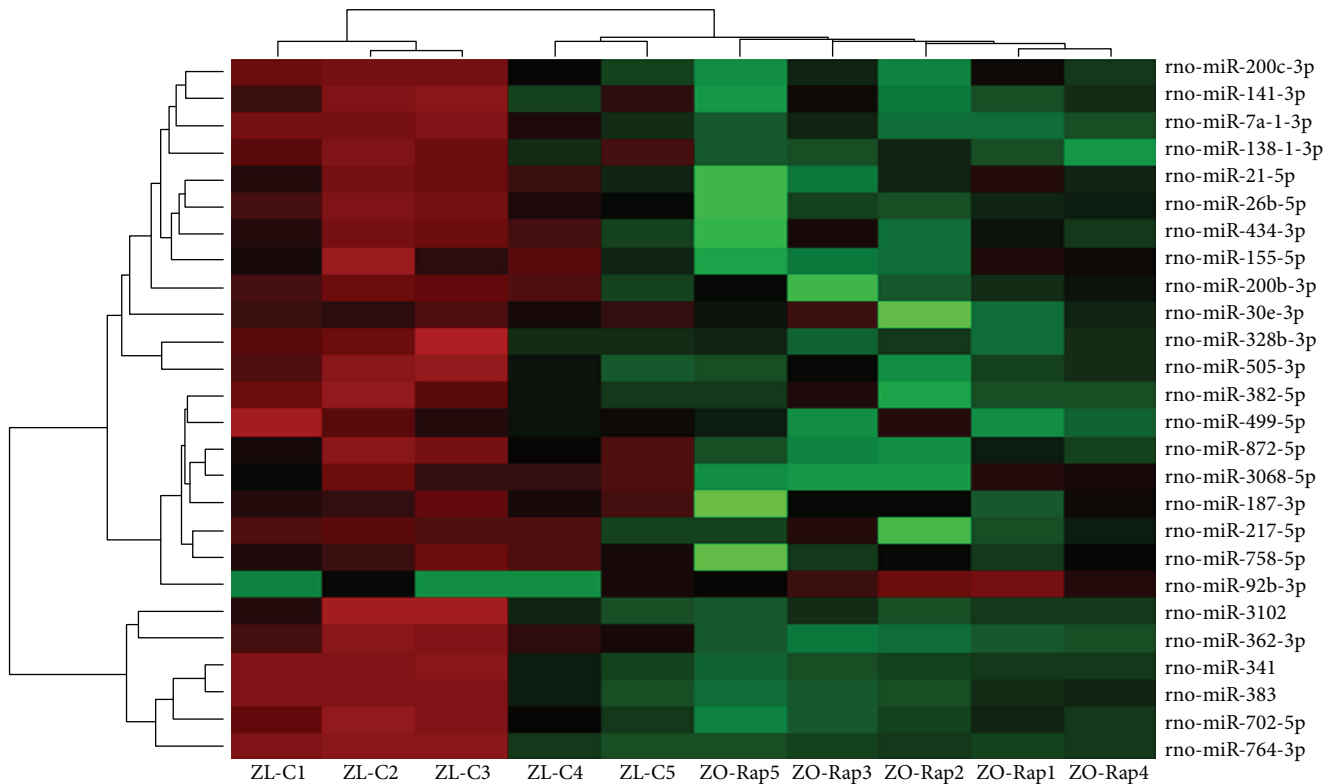
FIGURE 6: Hierarchal cluster heat map of differentially expressed cardiac miRNAs in ZL-C ($n = 5$) vs. ZO-Rap ($n = 5$) that exhibited a 2.25-fold change in expression in either direction. Red signals indicate lower expression levels, and green signals indicate higher expression levels.

(miR-34b-3p, miR-26b-3p, miR-140-5p, miR-155-5p, miR-21-5p, and miR-379-5p) exhibited differential expression consistent with the data obtained from microarray analysis.

4. Discussion

In this investigation, we have identified an identical subset of the cardiac miRNA transcriptome, comprised of 32 miRNAs, which are differentially expressed in the same direction in the hearts of diabetic ZO rats (ZO-C) and nondiabetic ZL rats treated with Rap (ZL-Rap). Since ZL rats did not develop diabetes after 3 months of Rap treatment [3] and Rap treatment is reported to mitigate aging [14–16], this high similarity between diabetes- and Rap-induced alterations to the cardiac miRNA transcriptome was surprising. Our recent findings that diabetes suppresses both inflammatory and anti-inflammatory intracardiac cytokines may shed some light in this regard [3]. As such, we posit that the similarity in the altered expression of identical miRNAs in the cardiac microRNA transcriptome between diabetic ZO-C and Rap-treated ZL rats might reflect that fact that both diabetes and Rap treatment cause significant immune suppression. Importantly, these studies also identified an identical subset of 17 miRNAs that exhibited increased expression in response to Rap treatment in both ZL and ZO hearts (Table 1). To our knowledge, this is the first evidence of a Rap-induced cardiac microRNA signature common to both healthy and diabetic hearts. It is noteworthy that most of these miRNAs seem to be associated with increasing CVD.

This information is clinically relevant and provides new targets for developing drugs that can be coadministered with Rap to reduce potential detrimental effects of long-term Rap treatment in patients with comorbidities.

Previous studies to identify Rap-induced changes in the miRNA transcriptome in cell models of tuberous sclerosis (TSC) and lymphangioleiomyomatosis (LAM) have identified microRNAs 29b, 21, 24, 221, 106a, and 199a as candidate "RapamiRs" [21]. Among these miRNAs, miR-21 and miR-29b were found to be differentially expressed in ZL-Rap. miR-21 is a key miRNA biomarker of diabetes that causes fibrosis and has been characterized as a "mechano-miR" due to its response to arterial stress and hypertension [59–64]. A direct connection between miR-21 and mTOR has also been reported since miR-21 promotes mTORC1-driven tumorigenesis [65]. Additionally, miR-155 has been identified as a potent autophagy inducer that targets the mTOR signaling pathway [66]. miR-29 family miRNAs are involved in pancreatic beta cell death and exacerbate cardiomyocyte loss [36–38, 67]. It was also reported that long-term Rap treatment induced the upregulation of miR-17–92 and related clusters and downregulation of tumor suppressor miRNAs (miR-7a, miR-706, and miR-320) in rapamycin-resistant tumors [68]. Consistent with this, 3-month Rap treatment increased miR-19a, a member of this cluster. However, Rap treatment actually increased miR-7a expression in ZL-C hearts.

Consistent with the pansuppression of cardiac cytokines in the ZO-C heart [3], we observed an increase in miRNAs

TABLE 5: Differentially expressed miRNAs in ZL-C versus ZO-C and ZL-C versus ZL-Rap pairwise comparisons that target differentially expressed cytokines in the corresponding pairwise comparisons and as determined by binding sites on the $3'$ untranslated region of their mRNAs by *in silico* analysis in the corresponding sets.

| miRNA | Targeted cytokines increased in | | | miRNA | Targeted cytokines | |
	ZO-C	ZL-Rap	Both		ZO-C	ZL-Rap
miR-21	CTACK, PDGFAA, CINC2	Notch2		miR-350		Notch2
miR-411	IL-2, IL-1α, TNF-α (2 sites)		IL-2, IL-10	miR-505	CINC2	Gas1
miR-7a*	PDGFAA	IL-2, decorin, IL-22	IL-10	miR-217		Notch 2, IL-1α
miR-384-5p		IL-2, IL-10, Notch2		miR-541		Notch 2, CINC2
miR-30e*		IL-22, Notch2	IL-10, IFNγ	miR-328b-3p		Notch 2, Gas1
miR-322			IFNγ	miR-29b		Notch 2
miR-382		Notch2	IFNγ, Prolactin	miR-455		Notch2
miR-742			IFNγ	miR-34b		Gas1
miR-539		IFNγ		miR-19a		Gas1
miR-329*	PDGFAA	Notch2	IL-10	miR-26b-3p		Gas1
miR-140		Notch2	IL-10, decorin	miR-429		Gas1, Tim1
miR-204	TNF-α	Gas1	IL-10	miR-128-1-5p		Gas1
miR-98	IL-1α		IL-10	miR-144		Gas1
miR-665		IL-10, Notch 2 (3 sites), Tim1		miR-2985		Gas1
miR-335		IL-10, Notch2, Tim 1		miR-301a		Prolactin
miR-495		IL-10 (2 sites), Notch 2, Gas1 (2 sites)		miR-362*		Tim1
miR-338*		IL-10, Notch2		miR-451*		Tim1
miR-200b, c			Decorin	miR-409-5p		Tim1
miR-223			Decorin	miR-218a		Tim1
miR-499		Decorin (2 sites), Gas1		miR-369-5p		Tim1
miR-542-3p		Decorin, Tim1		miR-434		IL-22
miR-138-1*	B7–1/CD80	Notch2		miR-379	TREM1, FGF-BP	
miR-196c	Decorin, TNF-α			miR-217	Notch 2, IL-1α	

∗ indicates a functional but nonpredominant miRNA as indicated by miRbase.

that target these cytokines in our *in silico* analysis (Table 5). miRNAs that have predicted binding sites on the $3'$ untranslated regions of mRNAs coding for the cytokines, CTACK, PDGFAA, CINC2, IL-2, IL-1α, TNF-α, IL-10, IFNγ, prolactin, and decorin were among the miRNAs that were upregulated in ZO-C hearts over 1.5 log2-fold. It is noteworthy that Notch2, decorin, and prolactin were also suppressed in ZO-Rap hearts [3]. However, in ZL-Rap hearts, while IL-2, GM-CSF, IL-10, and IFNγ were suppressed, decorin, Notch2, Gas1, prolactin, Tim1, IL-22, and TWEAK-R were upregulated by Rap treatment. Interestingly, analysis of predicted miRNA binding sites on the $3'$ untranslated regions of these mRNAs uncovered binding sites for many of the cardiac miRNAs that exhibited increased expression in ZL-Rap hearts. mRNAs encoding decorin, Notch2, Gas1, prolactin, Tim1, and IL-22 (but not TWEAK-R) carried binding sites for multiple miRNAs that were upregulated by Rap treatment. Although additional experiments are warranted to verify the validity of these miRNA binding sites, collectively, these observations suggest that the presence of a tightly regulated posttranscriptional gene expression pattern is present in the ZL-Rap heart for these cardiac cytokines.

We reported that in the ZO-Rap group, eight intracardiac cytokines were differentially expressed compared to ZO-C. However, only four miRNAs met the criteria of 1.5 log2-fold change in ZO-Rap compared to ZO-C. These miRNAs did not seem to have any predicted binding sites on the eight differentially expressed cytokines in the ZO-C heart. Importantly, cardiac miRNA transcriptome of ZO-C was already 80% similar to that of ZL-Rap and that may be why Rap treatment did not result in any additional major changes in their cardiac miRNA transcriptome that met the criteria of 1.5 log2-fold change.

4.1. Correlation of miRNA Transcriptome with Fibrosis. In

humans and animals alike, myocardial fibrosis is associated with nearly all forms of cardiovascular disease. Myocardial fibrosis is a condition of multiple etiologies, characterized by the transformation of cardiac fibroblasts to a myofibroblast phenotype. The cardiac remodeling that takes place
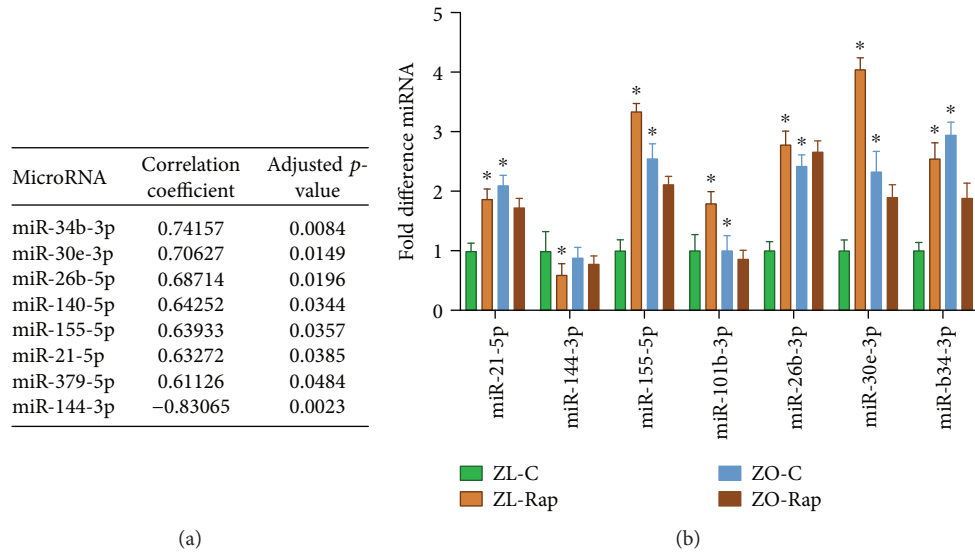
MicroRNA	Correlation coefficient	Adjusted p-value
miR-34b-3p	0.74157	0.0084
miR-30e-3p	0.70627	0.0149
miR-26b-5p	0.68714	0.0196
miR-140-5p	0.64252	0.0344
miR-155-5p	0.63933	0.0357
miR-21-5p	0.63272	0.0385
miR-379-5p	0.61126	0.0484
miR-144-3p	−0.83065	0.0023

(a)   (b)

FIGURE 7: (a) Correlation coefficients and p values of fibrotic scores and differentially expressed miRNA from all groups. (b) Comparative miRNA expression levels of several miRNAs that were associated with cardiac fibrosis scores. Data represents means ± SEM. $n = 4$ for all groups. $^*p < 0.05$ vs. the ZL-C group.

TABLE 6: miRNAs associated with cardiac fibrosis, their overall effect on fibrosis/TGF-β signaling, and the predicted target genes along the TGF-β signaling pathway.

MicroRNA	Assoc. with fibrosis (r^2, p value)	Fibrotic effect	Predicted gene targets in the TGF-beta signaling pathway[†]
hsa-miR-140	**0.6443, 0.034**	Anti [1]	*PITX2, BMP2*
hsa-miR-144	**−0.83, 0.002**	Anti [2, 3]	**ACVR2B**, *SMAD9, ROCK1, BMPR1B*, CDKN2B, ID4
hsa-miR-155	**0.639, 0.036**	Pro/anti* [4, 5]	*ACVR1C, BMPR2, SMAD5, SMAD2*, PPP2CA, *GDF6, SP1, RPS6KB1*
hsa-miR-21	**0.633, 0.039**	Pro [6–8]	**ACVR2A**, *BMPR2*, SMAD7, TGFB2
hsa-miR-26b	**0.687, 0.02**	Pro/anti* [9, 10]	*ACVR1C, BMPR1B, BMPR2, CREBBP, EP300*, IFNγ, INHBA, INHBB, *SMAD1, SMAD2*
hsa-miR-34b	**0.742, 0.008**	Pro [11]	**ACVR2A**, *ACVR1C*, FST, *SMAD5*, SMAD7, SMURF1, *RPS6KB1*

*Physiological conditionally based effects. [†]Emphasis indicates the predicted effect that miRNA inactivation of that gene would have on fibrosis. Italic emphasis refers to decreased fibrosis. Underlined emphasis refers to increased fibrosis. Bold emphasis refers to effect dependent on ligand milieu. ACVR1C: activin receptor type-1C; ACVR2A: activin receptor type-2A; ACVR2B: activin receptor type-2B; BMP2: bone morphological protein-2; BMPR2: bone morphological protein receptor type-2; CDKN2B: cyclin-dependent kinase inhibitor 2B; CREBBP: cAMP response element-binding protein; EP300: E1A-associated protein p300; FST: follistatin; GDF6: growth differentiation factor 6; ID4: inhibitor of DNA-binding protein 4; IFNγ: interferon-gamma; INHBA: inhibin beta A subunit; INHBB: inhibin beta B subunit; PITX2: paired-like homeodomain transcription factor 2; PPP2CA: protein phosphatase 2 catalytic subunit alpha; ROCK1: Rho-associated coiled-coil-containing protein; RPS6KB1: ribosomal protein S6 kinase B1; SMURF1: SMAD-specific E3 ubiquitin protein ligase 1; TGFB2: transforming growth factor beta 2.

during this phenotypic change is attributable to several pathologies including but not limited to LV dilation, ventricular stiffening, and cardiomyocyte death; all of which play pivotal roles in the progression to heart failure. We reported that ZL rats treated with rapamycin had greater degrees of cardiac fibrosis than untreated ZL rats. However, in ZO rats, treatment with rapamycin attenuated cardiac fibrosis [3]. Data presented here indicates that changes in the miRNA transcriptome induced by diabetes and Rap treatment shared significant similarities. Interestingly, while some of the miRNAs that were differentially expressed in ZO-C and ZL-Rap compared to ZL-C were profibrotic or increased in conditions of fibrosis, others were involved in suppressing fibrosis (Tables 2 and 3). This observation suggests that while cardiac fibrosis develops in response to diabetes (ZO-C) or Rap

treatment of healthy animals (ZL-Rap), a concurrent adaptive mechanism to regulate fibrosis via modulating miRNA transcriptome is also activated. Several signaling pathways have been implicated in the transformation of fibroblasts to myofibroblasts (fibrotic remodeling). Among the best understood of these signaling pathways are transforming growth factor-β, which is thought to be the primary regulatory pathway of pathological fibrosis [69, 70].

While some interspecies variation exists in the expression and target specificity of miRNA, previous studies have demonstrated high degrees of similarity [71, 72]. With the goal of exploring how our differentially expressed miRNA panel may be associated with cardiac fibrosis in humans, we utilized the DIANA software [30] and human orthologs for the miRNA that were significantly correlated with cardiac
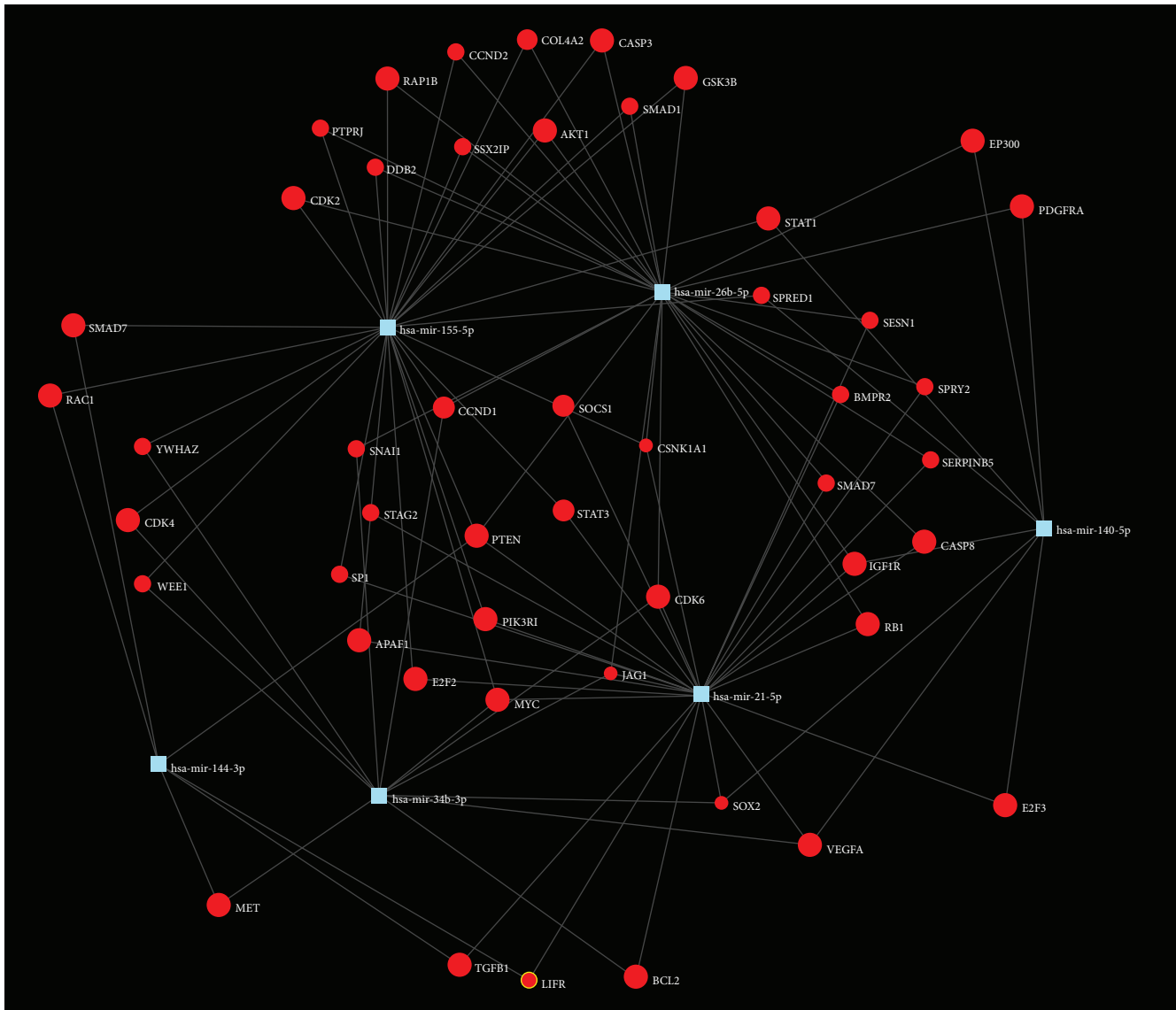
FIGURE 8: KEGG pathway enrichment analyses and subsequent identification of target genes along significantly enriched pathways associated with cardiac fibrosis. Generated using miRNet software.

fibrosis in our rats were used as input. KEGG analysis returned multiple interactions between our miRNA set and genes involved in the TGF-β signaling pathway (Table 6; Figure 8). Profibrotic TGF-β signaling involves numerous cell surface receptors in addition to TGF-β receptors, including those of the bone morphological protein and activin subfamilies (BMPRs and ACVRs, respectively). SMAD proteins are the primary signal transducers of these receptors and therefore are profibrotic with the exception of SMAD7 which inhibits the action of other SMADs. As with most biological pathways, the TGF-β pathway contains several internal feedback loops. Collectively, these data suggest that human orthologs of rat miRNAs that showed the highest correlation with cardiac fibrosis are involved in modulating the profibrotic TGF-β pathway.

In summary, the data presented here show that 47% of miRNA transcriptome activated in the hearts of healthy rats

in response to Rap treatment are identical to 80% of the miRNA transcriptome activated in diabetic rat hearts. In diabetic rat hearts, the miRNA transcriptome could have played a significant role in inducing the pansuppression of anti-inflammatory and proinflammatory intracardiac cytokines. However, while several miRNAs had predicted profibrotic effects, others had antifibrotic effects, suggesting that the miRNA transcriptome may serve as an adaptive mechanism to regulate the progression of cardiac fibrosis. Moreover, human orthologs of rat cardiac miRNAs that exhibited the highest correlation with cardiac fibrosis have the potential to modulate the profibrotic TGF-β pathway.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Supplementary Materials

Supplemental Table 1: differentially expressed miRNAs that were significant ($p < 0.05$) in ZO-C vs. ZL-C hearts. Supplemental Table 2: differentially expressed miRNAs that were significant ($p < 0.05$) in ZL-Rap vs. ZL-C hearts. Supplemental Table 3: differentially expressed miRNAs that were significant ($p < 0.05$) in ZO-Rap vs. ZO-C hearts. Supplemental Table 4: differentially expressed miRNAs that were significant ($p < 0.05$) in ZO-Rap vs. ZO-C hearts. Supplemental Table 5: KEGG pathway enrichment analysis of pathways corresponding to differentially expressed miRNA*. (Supplementary Materials)

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