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Identification of the microRNA Expression Profile in the Regenerative Neonatal Mouse Heart by Deep Sequencing

H. L. Liu · J. G. Zhu · Y. Q. Liu · Z. G. Fan ·
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Abstract MicroRNAs (miRNAs) are small noncoding RNAs that are involved in key biological processes, including development, differentiation, and regeneration. The global miRNA expression profile that regulates the regenerative potential of the neonatal mouse heart has not been reported. We performed deep sequencing to determine the genome-wide miRNA expression profile of the neonatal mouse heart at three key ages (1, 6, and 7 days). The miRNAs at least 1.4-fold differentially expressed between the three time points were selected for further analysis. Two miRNAs (mmu-miR-22-5p and mmu-miR-338-3p) were significantly upregulated, and nine miRNAs (mmu-miR-324-5p, mmu-miR-337-5p, mmu-miR-339-5p, mmu-miR-365-1-5p, mmu-miR-500-3p, mmu-miR-505-5p, mmu-miR-542-5p, mmu-miR-668-3p, and mmu-miR-92a-1-5p) were significantly downregulated in cardiac tissue of 7-day-old mice compared to 1- and 6-day-old mice. The expression patterns of five significantly different miRNAs were verified by quantitative real-time PCR. Furthermore, the potential targets of these putative miRNAs were suggested using miRNA target prediction tools. The candidate target genes are involved in the myocardial regenerative process, with a prominent role for the Notch signaling pathway. Our study provides a valuable resource for future investigation of the biological function of miRNAs in heart regeneration.

Keywords miRNA · Mouse · Heart · Regeneration · Deep sequencing

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Introduction

MicroRNAs (miRNAs) are an abundant class of 21–24 nucleotide noncoding RNAs that negatively regulate gene expression at the post-transcriptional level [1, 2]. At least 30 % of genes are predicted to be regulated by miRNAs. MiRNAs have been shown to play important roles in diverse biological processes, including proliferation, development, differentiation, and cell death [3]. Emerging evidence indicates that multiple miRNAs are dynamically regulated during tissue regeneration, including heart regeneration [4]. We sought to test whether miRNAs are differentially regulated during the process of regeneration in the neonatal mouse heart. Our results identify the expression profiles of miRNAs that are differentially regulated during heart regeneration. Several potential miRNA target genes involved in cardiac differentiation and the

Notch signaling pathway were predicted, suggesting a role for miRNAs in heart regeneration.

Urodele amphibians and *zebrafish* are known to possess the capacity for cardiac regeneration [5, 6]. Poss et al. [5] reported complete cardiac regeneration in adult *zebrafish* within 2 months after resecting 20 % of the ventricle. However, mature mammalian cardiac myocytes have long been believed to be terminally differentiated, as they are replaced by fibrous scar tissue without regenerative capability. A recent study shows that the neonatal mouse also retains a regenerative capacity over a small window of time (ages 1–6 days), but this capacity disappears by day 7 [7]. This suggests that the mammalian heart also possesses a limited capacity to regenerate. However, it is unknown whether miRNAs are involved in this process. In this study, to identify miRNAs that regulate gene expression during cardiac regeneration, we selected three key ages (1, 6, and 7 days) to perform miRNA screening by high-throughput deep sequencing in C57BL/6 mice. We validated five differentially regulated miRNAs using quantitative real-time PCR (qRT-PCR). Furthermore, we performed a comprehensive analysis of the differentially expressed miRNA target genes and pathways based on bioinformatic analysis.

Materials and Methods

Experimental Animals and Tissue Collection

Our study had been approved by the Animal Care and Use Committee of Nanjing Medical University, China. C57BL/6J male and female mice were housed under pathogen-free conditions. Animals were kept in individual cases in a temperature-controlled room (18–24 °C) with a 12-h light/dark cycle. C57BL/6J females were mated with males at the age of 6 months. The left ventricular apex was removed from 1-day-old, 6-day-old to 7-day-old mice [7]. Specimens (five mice per group) were snap frozen in liquid nitrogen and subsequently stored at −80 °C. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

MiRNA Sequencing and Sequence Analysis

The small RNAs were isolated using the previously described methods [8]. RNA fragments of 18–30 bases were collected using a Novex 15 % TBE-urea gel. Next, 5′ and 3′ adaptors were ligated to the ends of the small RNAs, and the RNA was converted to DNA by reverse transcription-PCR (RT-PCR). The RT-PCR products were purified on a 6 % TBE PAGE gel. The purified cDNA library was used directly for cluster generation and high-throughput sequencing with a Solexa sequencer (Huada Genomics Institute Co. Ltd, China) according to the manufacturer's protocol. The 50-nt sequence tags from high-throughput sequencing were screened for known miRNAs, novel miRNAs, and potential base edits of known miRNAs by the following procedure: (1) data cleaning was performed to remove low-quality reads and several varieties of contaminants, and the length distribution of these clean reads was then calculated. The length of small RNA is between 18 and 30 nt; (2) samples with common and specific sequences were grouped; (3) the expression and genomic distribution of small RNA tags were mapped by SOAP (Short Oligonucleotide Alignment Program); (4) alignments to known miRNA were performed; (5) an expression profile was generated for the known miRNAs; (6) small RNA tags were aligned to repeat-associated RNA to find matched tags in the samples; (7) the small RNA tags were screened for matches with rRNA, scRNA, snoRNA, snRNA and tRNA from GenBank, and matches to unannotated tags were eliminated; (8) the small RNA tags were screened for matches to sequences within Rfam, and matches to unannotated tags were eliminated; (9) small RNA tags were aligned to exons and introns of mRNA sequences to eliminate degraded fragments of mRNA in the small RNA tags; and (10) differential expression of known miRNAs was examined by Cluster analysis to annotate the clean tags into categories and to predict their mRNA targets.

Real-Time qPCR Validation of Differentially Expressed miRNAs

Real-time qRT-PCR was performed to confirm the differential expression of miRNAs by Solexa sequencing. Total RNA was isolated from neonatal mouse heart tissue using TRIzol (Invitrogen). A reverse transcription mixture was assembled containing 1 μg total RNA, 0.5 μl miRNA reverse primer (1 μM; Table 1), 0.3 μl RNase inhibitor (40 U/μl), 2 μl of 10× buffer, 2 μl RNasin (10 U/μl), and RNAse-free H_2O to a total volume of 20 μl. The reaction mixture was incubated at 16 °C for 30 min and then 42 °C for 40 min, followed by heat inactivation at 85 °C for 5 min. Real-time qRT-PCR was performed on an ABI 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) with 2.5 μl reverse transcription product, 0.5 μl forward and reverse primers each (Table 2), and 12.5 μl SYBR-Green real-time PCR master mix. The PCR cycling parameters were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s; and 78 °C for 20 s. The amount of mmu-miRNA was calculated relative to U6 small nuclear RNA (internal control) using the $2^{-\Delta\Delta Ct}$ method. All reactions were run at least in triplicate.

Table 1 RT primer sequences

MiRNA	RT primer
mmu-miR-22-5p	5′ GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTAAAGCT 3′
mmu-miR-338-3p	5′ GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCAACAAA 3′
mmu-miR-542-5p	5′ GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCGTGAC 3′
mmu-miR-339-5p	5′ GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCGTGAGCTC 3′
mmu-miR-668-3p	5′ GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGGTAGTG 3′
U6	5′ CGCTTCACGAATTTGCGTGTCAT 3′

Table 2 Primers for real-time RT-PCR

MiRNA	Primers	Product size (bp)
mmu-miR-22-5p	F: 5′ GGGAGTTCTTCAGTGGCA 3′	63
	R: 5′ CAGTGCGTGTCGTGGAGT 3′	
mmu-miR-338-3p	F: 5′ GGGTCCAGCATCAGTGA 3′	65
	R: 5′ CAGTGCGTGTCGTGGAGT 3′	
mmu-miR-542-5p	F: 5′ GGCTCGGGGATCATCAT 3′	64
	R: 5′ CAGTGCGTGTCGTGGAGT 3′	
mmu-miR-339-5p	F: 5′ GGGTCCCTGTCCTCCA 3′	66
	R: 5′ TGCGTGTCGTGGAGTC 3′	
mmu-miR-668-3p	F: 5′ TGTCACTCGGCTCGG 3′	61
	R: 5′ TGCGTGTCGTGGAGTC 3′	
U6	F: 5′ GCTTCGGCAGCACATATA CTAAAAT 3′	89
	R: 5′ CGCTTCACGAATTTGCGTG TCAT 3′	

Target Gene Prediction

Predicted targets for mmu-miR-532-5p, mmu-miR-22-5p, mmu-miR-338-3p, mmu-miR-542-5p, mmu-miR-339-5p, and mmu-miR-668-3p were analyzed using miRanda (http://microrna.sanger.ac.uk/sequences/index.shtml) and DIANA Lab (http://diana.cslab.ece.ntua.gr).

Statistical Analysis

Putative miRNA candidates were selected according to the following criteria: (1) fold-change was calculated based on the ratio of normalized counts between different time points; (2) statistical analyses were performed using one-way analysis of variance (ANOVA), and t tests or Student's-$t$tests were performed with a correction for multiple comparisons; and (3) values were expressed as mean $\pm$ SEM. $P < 0.05$ was considered to indicate a statistically significant result.
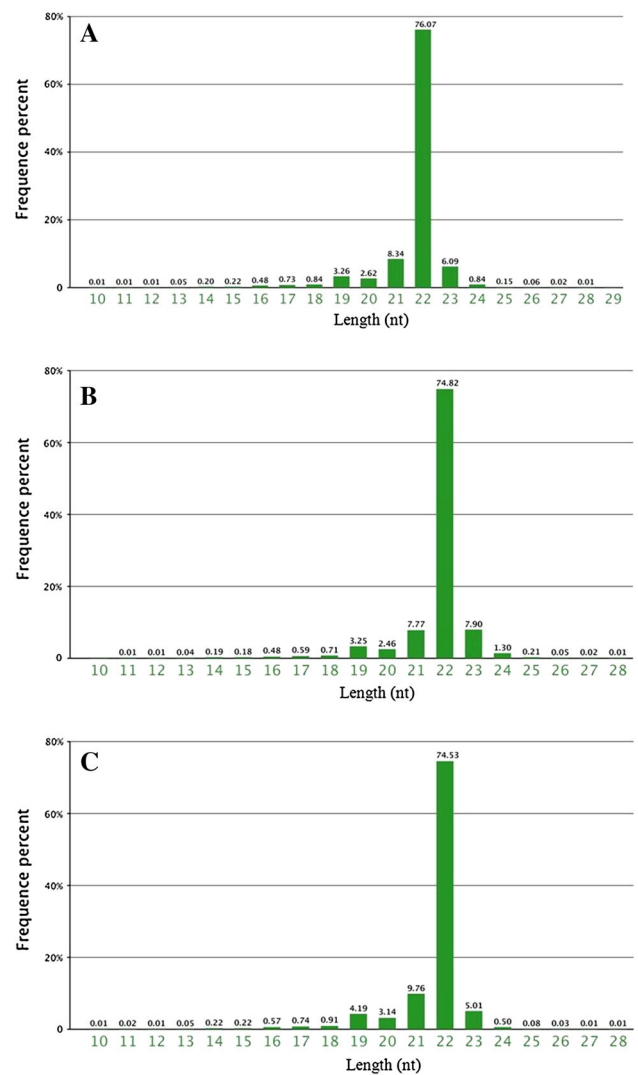

Fig. 1 Length distribution and abundance of the sequences in the three groups of mouse cardiac tissue. The distribution of different length sequences is shown for **a** the 1-day-old group; **b** the 6-day-old group; and **c** the 7-day-old group

Table 3 Summary of common and specific sequences between the 1- and 6-day-old mouse cardiac tissue

Class	Unique sRNAs	Percent (%)	Total sRNAs	Percent (%)
Total sRNAs	198835	100	21778584	100
Days 1–6	33464	16.83	21589004	99.13
Day 1-specific	95748	48.15	112005	0.51
Day 6-specific	69623	35.02	77575	0.36

Table 4 Summary of common and specific sequences between the 1- and 7-day-old mouse cardiac tissue

Class	Unique sRNAs	Percent (%)	Total sRNAs	Percent (%)
Total sRNAs	204188	100	21618007	100
Days 1–7	34221	16.76	21422621	99.10
Day 1-specific	94991	46.52	110746	0.51
Day 7-specific	74976	36.72	84640	0.39

Table 5 Summary of common and specific sequences between the 6- and 7-day-old mouse cardiac tissue

Class	Unique sRNAs	Percent (%)	Total sRNAs	Percent (%)
Total sRNAs	181190	100	21826663	100
Days 6–7	31094	17.16	21654857	99.21
Day 6 specific	71993	39.73	81761	0.37
Day 7 specific	78103	43.11	90045	0.41

Results

Overview of the Solexa Sequencing Data

To identify miRNA expression profiles in the regenerative heart, deep sequencing of small RNA was performed using Solexa technology, and raw reads were obtained. After eliminating low-quality tags, contaminants, and trimming adaptors, we obtained 10784964, 10993620, and 10833043 clean reads from 1-, 6-, and 7-day-old groups, respectively. The length distribution of small RNA was used to analyze the composition of small RNA (Fig. 1). Most of the mature miRNAs were between 19 and 23 nucleotides (nt) for all three groups. The majority of small RNAs from the libraries were 22 nt long. The common and specific tags for the libraries, including a summary of the numbers of unique tags and total tags, are listed in Tables 3, 4, and 5. The results indicate that most of the miRNA sequences were conserved between all of the groups, with the number of group-specific sequences being much smaller.

MiRNA Expression Profiles

We used Solexa deep sequencing technology to evaluate the expression profiles of miRNAs from the cardiac tissue of the three groups. Comparison analysis showed the differential expression of microRNAs in myocardial tissues of 1-, 6-, and 7-day-old mice. Although these groups expressed many of the same miRNAs (Tables 3, 4, 5), the miRNA expression pattern within each group was found to vary significantly (Fig. 2). The miRNAs with at least 1.4-fold difference between all three time points were selected for further analysis. Specifically, we identified four miRNAs with increased expression and 22 with decreased expression in cardiac tissue of 6-day-old mice compared to 1-day-old mice (Table 6), eight miRNAs with increased expression and 40 miRNAs with decreased expression in cardiac tissue of 7-day-old mice compared to 1-day-old mice (Table 7), and nine miRNAs with increased expression and 10 miRNAs with decreased expression in cardiac tissue of 7-day-old mice compared to 6-day-old mice (Table 8).

Validation of Differently Expressed miRNAs Using Real-Time qRT-PCR

To validate the Solexa deep sequencing, real-time qRT-PCR analysis was performed. Two upregulated miRNAs (mmu-miR-22-5p and mmu-miR-338-3p) and three downregulated miRNAs (mmu-miR-542-5p, mmu-miR-339-5p, and mmu-miR-668-3p) were selected, and expression was examined in cardiac tissue of 7-day-old mice compared to 1- and 6-day-old mice. The expression data obtained by real-time qRT-PCR analysis were consistent with the high-throughput sequencing results (Fig. 3).

Prediction of miRNA Target Genes

To better understand the biological functions of differently expressed miRNAs, the putative target sites of these miRNAs were predicted using online miRNA target prediction tools (http://microrna.sanger.ac.uk/sequences/index.shtml and http://diana.cslab.ece.ntua.gr). Among the miRNA target genes, we found that *GATA1*, *TBX5*, *Baf60c*, *DLK1*, *Dvl1*, and *LFNG* were targets of mmu-miR-532-5p, mmu-miR-22-5p, mmu-miR-338-3p, mmu-miR-542-5p, mmu-miR-668-3p, and mmu-miR-339-5p, respectively.

Discussion

It is well established that urodele amphibians and teleost fish have life-long myocardial regeneration capacity [6, 9]. A recent study found that the neonatal mouse also retains

Fig. 2 Hierarchical miRNA clustering of the 1-, 6-, and 7-day-old groups (P1 represents 1-day-old group; P2 represents 6-day-old group; P3 represents 7-day-old group). Distinguishable miRNA expression profiles are observed between the three groups. *Red* indicates high relative expression, whereas *green* indicates low relative expression (Color figure online)
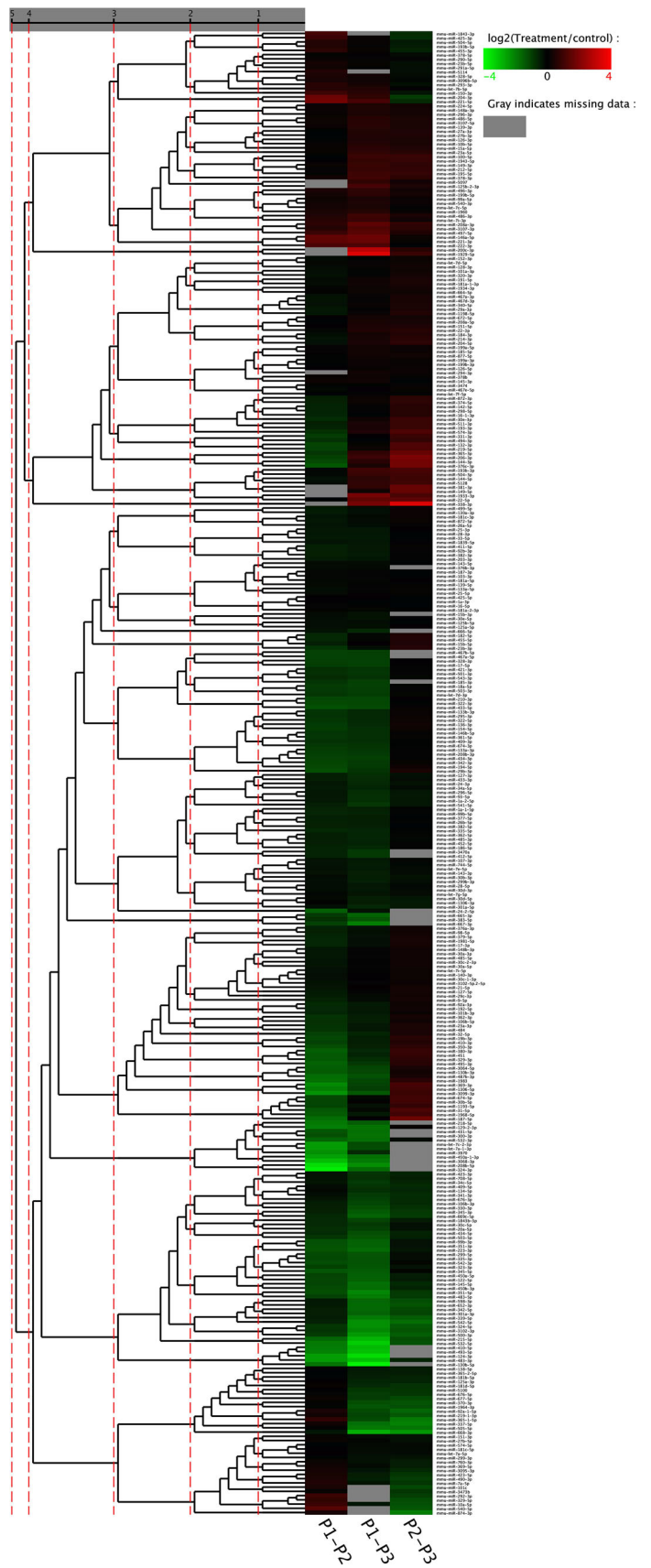
Table 6 Differentially expressed miRNAs in 6-day-old versus 1-day-old mouse cardiac tissue

MiRNA	Regulation	Log2	P-value
mmu-miR-146a-5p	Up	1.50	0.0027
mmu-miR-204-3p	Up	1.68	0.0039
mmu-miR-221-3p	Up	1.47	1.2185E-212
mmu-miR-221-5p	Up	1.82	2.4324E-06
mmu-let-7a-1-3p	Down	−2.44	0.0022
mmu-let-7c-2-3p	Down	−2.44	0.0022
mmu-miR-129-2-3p	Down	−1.98	7.7267E-13
mmu-miR-1306-5p	Down	−1.99	2.2359E-06
mmu-miR-130b-3p	Down	−1.44	5.1565E-22
mmu-miR-1983	Down	−1.76	1.6869E-24
mmu-miR-208b-5p	Down	−3.48	9.1318E-08
mmu-miR-215-5p	Down	−1.58	1.3079E-157
mmu-miR-3064-5p	Down	−1.49	0.0007
mmu-miR-3068-3p	Down	−2.96	4.0116E-05
mmu-miR-3099-3p	Down	−2.30	1.1474 E-05
mmu-miR-324-3p	Down	−3.72	0.0008
mmu-miR-329-3p	Down	−1.41	8.5894E-17
mmu-miR-369-3p	Down	−2.17	5.4937E-05
mmu-miR-380-3p	Down	−1.50	0.0050
mmu-miR-3970	Down	−2.24	0.0066
mmu-miR-450a-1-3p	Down	−2.53	0.0012
mmu-miR-483-3p	Down	−2.45	1.3867E-59
mmu-miR-487b-3p	Down	−1.70	0.0004
mmu-miR-493-5p	Down	−2.02	0.0063
mmu-miR-495-3p	Down	−1.54	2.2417E-07
mmu-miR-532-5p	Down	−1.90	1.0675E-76

Table 7 Differentially expressed miRNAs in 7-day-old versus 1-day-old mouse cardiac tissue

| MiRNA	Regulation	Log2	P-value
mmu-miR-1929-5p	Up	3.45	0.0035
mmu-miR-146a-5p	Up	1.71	0.0004
mmu-miR-1933-3p	Up	1.86	0.0359
mmu-miR-200c-3p	Up	2.9	0.0013
mmu-miR-221-3p	Up	1.63	5.67E-272
mmu-miR-22-5p	Up	1.5	2.57E-08
mmu-miR-338-3p	Up	1.86	0.0359
mmu-miR-208a-3p	Up	1.45	1.84E-18
mmu-miR-122-5p	Down	−1.55	1.42E-34
mmu-miR-124-3p	Down	−3.46	0.0033
mmu-miR-129-2-3p	Down	−1.81	6.46E-08
mmu-miR-130b-5p	Down	−3.81	0.0005
mmu-miR-145-5p	Down	−1.74	1.25E-64
mmu-miR-208b-5p	Down	−2.24	2.38E -05
mmu-miR-215-5p	Down	−2.81	2.9643E-323
mmu-miR-223-3p	Down	−1.68	0.0005
mmu-miR-300-3p	Down	−1.81	0.0187
mmu-miR-301a-3p	Down	−1.56	4.51E-06
mmu-miR-3068-3p	Down	−1.94	0.0013
mmu-miR-3102-3p	Down	−2.13	3.64E-23
mmu-miR-324-3p	Down	−1.7	0.0301
mmu-miR-324-5p	Down	−2.27	4.85E-14
mmu-miR-337-5p	Down	−1.75	0.0001
mmu-miR-339-5p	Down	−1.93	0.0039
mmu-miR-342-5p	Down	−1.63	6.16E-09
mmu-miR-345-3p	Down	−1.43	1.03E-50
mmu-miR-345-5p	Down	−1.45	3.88E-14
mmu-miR-351-3p	Down	−1.56	3.62E-98
mmu-miR-351-5p	Down	−2.13	2.64E-104
mmu-miR-410-5p	Down	−2.91	0.0012
mmu-miR-431-5p	Down	−1.74	0.0057
mmu-miR-450a-5p	Down	−1.51	6.39E-26
mmu-miR-450b-3p	Down	−1.71	3.21E-83
mmu-miR-483-3p	Down	−3.02	1.92E-73
mmu-miR-483-5p	Down	−1.95	0
mmu-miR-493-5p	Down	−3	0.0007
mmu-miR-500-3p	Down	−2.42	5.25E-10
mmu-miR-505-5p	Down	−1.92	9.50E-15
mmu-miR-532-3p	Down	−1.75	3.54E-25
mmu-miR-532-5p	Down	−3.2	4.84E-138
mmu-miR-542-5p	Down	−2.24	3.75E-10
mmu-miR-598-3p	Down	−1.62	2.18E-13
mmu-miR-652-3p	Down	−1.68	2.93E-09
mmu-miR-665-3p	Down	−1.59	0.0259
mmu-miR-667-3p	Down	−2	0.0207
mmu-miR-668-3p	Down	−2.74	0.0002
mmu-miR-669c-5p	Down	−1.56	5.50E-12
mmu-miR-99b-3p	Down	−1.54	1.54E-24

capacity for cardiac regeneration, but that this ability persists only in the first week of life [7]. The molecular mechanisms underlying this phenomenon have not been elucidated. MiRNAs have recently been shown to play a vital role in cardiac regeneration [10, 11]. We collected myocardial tissue from 1-, 6- (turning point) and 7-day-old mice, and used deep sequencing technology to determine the comprehensive miRNA expression profiles in myocardial tissue of these three groups. mmu-miR-532-5p expression gradually reduced during the period of neonatal heart development. Based on our findings from miRNA target prediction software, *GATA1* was identified as a target of mmu-miR-532-5p. *GATA1* is a member of the GATA family of zinc finger transcription factors, which is essential for red blood cell and megakaryocyte maturation [12]. Caprioli et al. [13] showed that the differentiation of stem cells into hematopoietic cells is suppressed at low levels of GATA1 expression, but that this transcriptional regulator may also promote the differentiation of embryonic stem

Table 8 Differentially expressed miRNAs in 7-day-old versus 6-day-old mouse cardiac tissue

MiRNA	Regulation	Log2	P-value
mmu-miR-144-3p	Up	1.82	0.0001
mmu-miR-149-5p	Up	1.6	0.0456
mmu-miR-187-5p	Up	1.72	0.0285
mmu-miR-206-3p	Up	1.96	5.42E-47
mmu-miR-22-5p	Up	1.49	2.67E-08
mmu-miR-338-3p	Up	3.48	0.0031
mmu-miR-365-3p	Up	1.6	2.25E-05
mmu-miR-376c-3p	Up	1.79	0.0018
mmu-miR-381-3p	Up	1.6	0.0456
mmu-miR-324-5p	Down	−1.41	4.96E-05
mmu-miR-337-5p	Down	−2.04	2.65E-06
mmu-miR-339-5p	Down	−1.46	0.0447
mmu-miR-365-1-5p	Down	−1.92	9.38E-07
mmu-miR-500-3p	Down	−1.48	0.0012
mmu-miR-505-5p	Down	−1.84	1.49E-13
mmu-miR-542-5p	Down	−1.71	1.32E-05
mmu-miR-668-3p	Down	−2.39	0.0028
mmu-miR-874-3p	Down	−1.97	0.0229
mmu-miR-92a-1-5p	Down	−1.6	0.0003
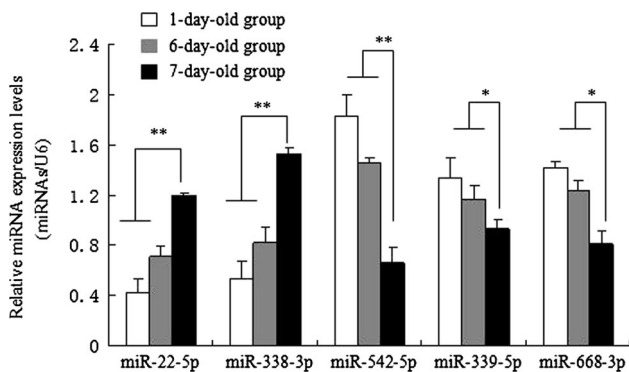
Fig. 3 Validation of microarray data using real-time qRT-PCR. The real-time qRT-PCR analyses were repeated three times for every miRNA. *Error bars* indicate standard error. * $p < 0.05$. ** $p < 0.01$

cells into myocardial cells. We hypothesize that due to reduction in mmu-miR-532-5p expression, the expression of *GATA1* in 7-day-old mice cardiac tissue gradually increases and thereby inhibits the regeneration of myocardial cells.

Two upregulated miRNAs (mmu-miR-22-5p and mmu-miR-338-3p) were differentially expressed in 7-day-old mice, compared to 1- and 6-day-old mice. A recent study in LO2/HBx-d382 cells indicated that miR-338-3p expression inhibits cell proliferation [14]. Given that the cardiac regenerative capacity is lost in 7-day-old mice, where mmu-miR-338-3p was expressed at the highest levels, upregulation of mmu-miR-338-3p could inhibit myocardial

proliferation. Furthermore, the predicted target genes of mmu-miR-22-5p and mmu-miR-338-3p are *TBX5* and *Baf60c*, respectively. TBX5 and Baf60c can direct ectopic differentiation of mouse mesoderm into beating cardiomyocytes, and TBX5 is essential for differentiation into contracting cardiomyocytes [15]. Ieda et al. [16] reported that GATA4, Mef2c, and TBX5 efficiently reprogrammed fibroblasts directly into functional cardiomyocytes. Based on our data and the reported role of these genes in cardiomyocyte differentiation, we hypothesize that mmu-miR-22-5p and mmu-miR-338-3p may reduce the expression of *TBX5* and *Baf60c*, preventing the ability to regenerate the myocardium in 7-day-old mice.

A number of previous studies have identified the signaling pathways critical for cardiomyocyte proliferation, apoptosis, and regeneration [17–19]. In the present study, the target genes of the significantly downregulated miRNAs (mmu-miR-542-5p, mmu-miR-339-5p, and mmu-miR-668-3p) in cardiac tissue of 7-day-old mice included *DLK1*, *LFNG,* and *Dvl1*. These genes play an important role in regulating the Notch signaling pathway [20–22], providing support for a potential role for the Notch signaling pathway in the activation of heart regeneration [23].

In summary, using high-throughput Solexa sequencing, we classified a large number of miRNAs in the 1- to 7-day-old neonatal mouse heart. Importantly, we identified a series of miRNAs that are differentially expressed at these three time points. Cardiac regeneration is a complex biological process. More work will be needed to determine the functions, relationships, and mechanisms of action of miRNAs in cardiac regeneration.

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