Differential expression of microRNAs in cardiac myocytes compared to undifferentiated P19 cells

DE-LIANG HU[1*], YAO-QIU LIU[1*], FU-KUN CHEN[1], YAN-HUI SHENG[1], RONG YANG[1], XIANG-QING KONG[1], KE-JIANG CAO[1], JIN-SONG ZHANG[2] and LING-MEI QIAN[1]

Departments of [1]Cardiology and [2]Emergency, First Affiliated Hospital of Nanjing Medical University, 210029 Nanjing, P.R. China

Received January 21, 2011; Accepted March 10, 2011

DOI: 10.3892/ijmm.2011.664

Abstract. microRNA (miRNA) expression is tightly controlled in a tissue-specific and developmental stage-specific manner; some are highly and specifically expressed in cardiovascular tissues. miRNA expression profiling, using miRNA microarrays facilitates studying the biological function of miRNAs. We investigated changes in miRNA expression profiles during differentiation of P19 cells into cardiac myocytes in order to elucidate the mechanisms of heart development. The morphology of P19 cells during differentiation was observed using an inverted microscope. Western blot analysis was performed to detect cardiac troponin I (cTnI) expression. Total RNA was extracted from P19 cells for microarray and real-time quantitative reverse transcription-polymerase chain reaction (real-time qRT-PCR) analyses to determine the miRNA expression profile. The miRNA microarray revealed differential expression of 49 miRNAs, of which 26 were down-regulated and 23 were up-regulated in differentiated cardiac myocytes, compared to normal P19 cells. This was confirmed by real-time qRT-PCR. We also utilized target prediction analysis to identify gene targets. Some miRNAs may have important roles in cardiac development and congenital heart defects (CHDs). Further analysis of miRNA function to confirm their target genes during cardiac development will determine the potential for novel miRNA-based therapeutic strategies.

Introduction

Animal studies and advances in human genetics have demonstrated the precise regulation of key molecular pathways during embryonic development. This is particularly true for the cardiovascular system, in which haploinsufficiency of essential genes often causes cardiac malformations (1), the most common birth defects in humans occurring in roughly 1% of newborns (2) and resulting from defects in cell lineage or morphogenetic decisions. Recently, a class of small non-coding RNAs, called microRNAs (miRNAs), has emerged as a central regulator of many cardiogenic processes. miRNAs are involved in various cellular processes, such as proliferation, differentiation, apoptosis, stress response, oncogenesis, tumor suppression (3-8) and developmental timing (9) in both animals and in humans. miRNAs are derived from characteristic hairpins in primary transcripts through two sequential cleavages, Drosha and Dicer (10). They regulate gene expression post-transcriptionally by binding to the target mRNA, causing translational inhibition or degradation (11-13). Incomplete base pairing usually occurs at the 3'-untranslated region (3'UTR) of the target mRNA and at the seed sequence, the first 6-8 bases from the 5'-end of the mature miRNA. Although miRNAs most commonly lead to decreased gene expression, they can also induce up-regulation of genes by negatively modulating the expression of inhibitory genes. Alternatively, down-regulation of miRNAs may also induce up-regulation of genes that have been previously suppressed (14). miRNAs regulate diverse biological processes, including tissue differentiation and maintenance, and contribute to varied disease processes (15,16).

Identification of miRNAs expressed in specific cardiac cell types could define important regulatory roles for these miRNAs during cardiomyocyte differentiation, cell cycle progression, conduction, and adult cardiac hypertrophy. P19 cells, isolated from an experimental embryo-derived mouse teratocarcinoma, are multipotent and can differentiate into cell types representing all 3 germ layers. In the presence of DMSO, P19 cells differentiate into cardiac myocytes with embryoid body formation (17). Because they can differentiate into cardiac myocytes, P19 cells are used to investigate cardiac-specific transcription factors and upstream signaling pathways in cardiac cell differentiation (18-20). P19 cells are an excellent model system for studying myocardial electrophysiological differentiation regulation at the molecular and functional levels (21).

Given that P19 cells can develop into cardiac myocytes, elucidation of the regulatory mechanisms controlling their self-renewal, proliferation and differentiation could provide

Correspondence to: Dr Ling-Mei Qian, Department of Cardiology, First Affiliated Hospital of Nanjing Medical University, 210029 Nanjing, P.R. China
E-mail: lmqian@njmu.edu.cn

Dr Jin-Song Zhang, Department of Emergency, First Affiliated Hospital of Nanjing Medical University, 210029 Nanjing, P.R. China
E-mail: zhangjso@sina.com

*Contributed equally

Key words: microRNAs, cardiac myocytes, microarrays

Table I. RT primers sequences.

Gene name	RT primers
mmu-mir-690	5'-CGTCGCGGCATCGAGTGGAGCAGACCGACAGCGCGACGTTTGGTTGT-3'
mmu-mir-25	5'-CGTCGCGGCATCGAGTGGAGCAGACCGACAGCGCGACGTCAGACC-3'
mmu-mir-92a	5'-CGTCGCGGCGGTCGGACTACATCATCGCGACGCAGGCC-3'
mmu-mir-302b	5'-CGTCGCGGCGGTCGGACTACATCATCGCGACGCTACTA-3'

Table II. Primers for real-time RT-PCR.

Gene name	Primers	Ta (˚C)
mmu-mir-690	P5: 5'-GGCAAAGGCTAGGCTCACA-3' P3: 5'-GCATCGAGTGGAGCAGACC-3'	55
mmu-mir-25	P5: 5'-CCCATTGCACTTGTCTCGG-3' P3: 5'-GCATCGAGTGGAGCAGACC-3'	55
mmu-mir-302b	P5: 5'-GGTAAGTGCTTCCATGTTT-3' P3: 5'-GCGGTCGGACTACATCAT-3'	55
mmu-mir-92a	P5: 5'-GGTATTGCACTTGTCCCG-3' P3: 5'-GCGGTCGGACTACATCAT-3'	55
U6	P5: 5'-CAGGGGCCATGCTAAATCTTC-3' P3: 5'-CTTCGGCAGCACATATACTAAAAT-3'	55

new venues to explore the underlying mechanisms of heart development. miRNA microarrays are a high throughput global analysis tool for miRNA expression profiling, which will facilitate the study of miRNA biological function. In this report, we determine miRNA expression in normal P19 cells and cardiac myocytes using a microarray. We validated the microarray results using real-time quantitative reverse transcription PCR (real-time qRT-PCR) for specific differentially expressed miRNAs.

Materials and methods

Cell culture. P19 cell culture and induction of differentiation. P19 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultivated as aggregates for 4 day in 1% α-minimal essential medium (α-MEM, Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco-BRL) and 1% dimethylsulfoxide (DMSO, Sigma, St. Louis, MO) in bacteriological dishes in a 5% CO_2 atmosphere at 37˚C (19). The medium was replaced every 2 days. On day 4, the cell aggregates were transferred to cell culture flasks. Cells were harvested on differentiation day 0 and day 10. The morphological changes in P19 cells were examined under an inverted microscope (Nikon Eclipse TE300, Tokyo, Japan) equipped with phase-contrast objectives and a digital camera (Nikon E4500).

Western blotting. Cultured cells were directly transferred to tubes containing lysis buffer (1% Triton X-100, 50 mmol/l Tris-HCl, 0.2% SDS, 0.2% sodium deoxycholate, and 1 mmol/l EDTA at pH 7.4) and vortexed briefly. The lysate supernatant was collected after centrifugation at 15,200 x g for 15 min at 4˚C. Protein concentration determinations using a protein assay reagent kit, and Western blotting using a monoclonal rabbit anti-cardiac troponin I (anti-cTnI) antibody (Chemicon, San Diego, CA, USA) and a monoclonal rabbit anti-GAPDH antibody (Proteintech Group, Inc., China), were performed as previously described (22).

miRNA microarray. Total RNA extraction was performed with the mirVana extraction kit (Ambion, Austin, TX), following the manufacturer's protocol. The quality of the extracted RNA was assessed by an electropherogram and gel analysis, and an RNA integrity number (RIN) was calculated by the Agilent Bioanalyzer™ for each RNA sample. The microarray assay was performed using a service provider (LC Sciences, Houston, TX). The assay was performed with a 2-5 μg total RNA sample, which was size fractionated using a YM-100 Microcon centrifugal filter (Millipore) and the isolated small RNAs (<300 nt) were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for subsequent fluorescent dye staining. Hybridization was performed overnight on a microParaflo microfluidic chip using a microcirculation pump (Atactic Technologies, Houston, TX) (23,24). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to the target microRNA (from miRBase, Release 14.0, http://microrna.sanger.ac.uk/sequences) or other RNA (control) and a polyethylene glycol spacer segment to extend the coding segment away from the

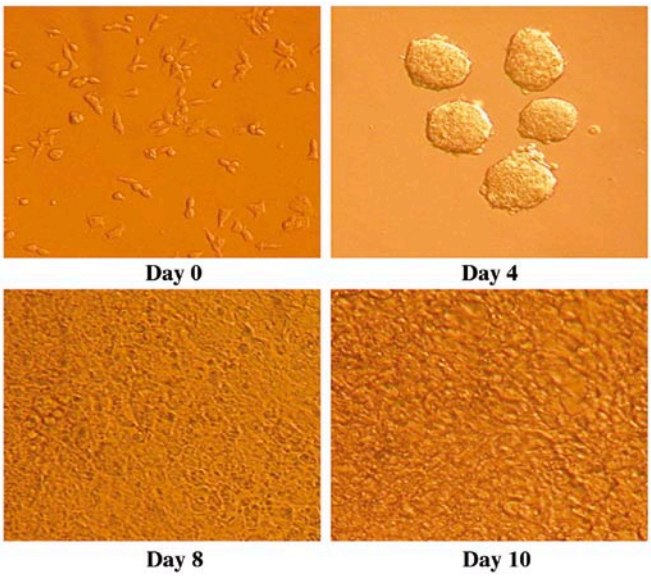

Figure 1. Morphology of P19 cells during differentiation. P19 cells were aggregated for 4 days and the colonies of beating cells were observed on day 10 under an inverted microscope as described in Materials and methods.

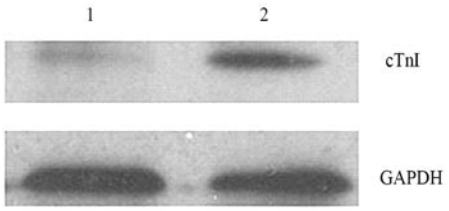

Figure 2. Expression of cTnI protein in P19 cells. Total proteins were isolated from P19 cells and analyzed by western blotting. Lane 1, day 8; Lane 2, day 10. The experiment was repeated 3 times with consistent results.

substrate. The detection probes were synthesized *in situ* using photogenerated reagent (PGR) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. For hybridization we used 100 μl 6X SSPE buffer (0.90 mol/l NaCl, 60 mmol/l Na_2HPO_4, 6 mmol/l EDTA, pH 6.8) containing 25% formamide at 34˚C. After hybridization, miRNAs were detected by fluorescent labeling using tag-specific Cy5 dyes. Hybridization images were obtained using a laser scanner (GenePix 4.1, Molecular Device) and digitized using the Array-Pro image analysis software (Media Cybernetics).

Data were analyzed by first subtracting the background and then normalizing the signals using a Lowess filter (locally-weighted regression) (25). In order for a transcript to be listed as detectable at least two conditions needed to be met: the signal intensity needed to be >3X (background standard deviation) and the spot CV <0.5. The CV was calculated by the ratio of (standard deviation)/(signal intensity). When repeating probes were present on an array, a transcript was listed as detectable only if the signals from at least 50% of the repeating probes were above detection level.

Experimental validation. Total RNA was isolated from cultured P19 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA). Single-strand cDNA was synthesized as follows: the reverse transcription mixture contained 2 μl total RNA, 1 μl mmu-miRNA reverse primer (Table I), 1 μl ReverTra Ace (Toyobo FSK100, Osaka, Japan), 4 μl 5X buffer, 2 μl dNTP mix (10 mmol/l), 1 μl RNasin, 1 μl random primer and 8 μl RNAse-free H_2O, in a 20 μl total volume. The reaction was performed at 30˚C for 10 min and at 42˚C for 20 min, followed by heat inactivation at 99˚C for 5 min and then at 4˚C for 5 min. For real-time PCR, 2 μl cDNA was added to 28 μl master mix containing 0.5 μl SYBR-Green (Applied Biosystems, Foster City, CA) and 0.5 μl reverse and forward primers. cDNA was amplified for 35 cycles with the Applied Biosystems 7300 real-time PCR system. The primer sequences used are listed in Table II. The amount of PCR product was calculated from the threshold cycle (Ct), the amplification cycle at which the emission intensity of the product rises above a set threshold level.

Target prediction analysis. We used TargetScan 5.1 (http://www.targetscan.org/) to perform bioinformatics-based target prediction analysis.

Statistical analysis. Data were analyzed using the Student's t-test with the SPSS 15.0 statistical package. A P-value <0.05 was considered significant.

Results

P19 cell differentiation. Cell aggregation is a requirement for DMSO-induced P19 cell differentiation into cardiac myocytes. Efficient P19 cell differentiation depends on the prior formation of non-adhering aggregates (18). To investigate how P19 cells can be differentiated into cardiac cells, we examined morphological changes in P19 cells. P19 cells were aggregated for 4 days and the colonies of beating cells were observed on day 10 (Fig. 1). Western blotting was used to detect cTnI (a myocyte differentiation marker) protein expression (Fig. 2). cTnI expression was much higher on day 10 compared to day 8. The beating cell colonies generated on day 10 of P19 cell differentiation, as well as the high cTnI protein expression, indicated that the cells had differentiated into cardiac myocytes.

miRNA microarray. We performed microarray analysis on three replicate samples of cells on day 0 and day 10 of DMSO exposure. Patterns of differentially expressed miRNAs were observed (Fig. 3). Those with a signal value >500, with a P-value <0.01, were selected as the high signal group (differentially expressed). As summarized in Table III, a total of 49 differentially expressed miRNAs in cardiac myocytes (day 10) compared to normal P19 cells (day 0) were identified. Among them, 23 were up-regulated and 26 were down-regulated.

Validation of differentially expressed miRNAs. To validate the microarray results, we performed real-time qRT-PCR expression analysis on the cells at day 0 and day 10. miRNAs regulate gene expression post-transcriptionally by binding to the target mRNA, causing translational inhibition or degradation. In order to more closely examine target genes that promote differentiation, the expression of 4 miRNAs down-regulated (mir-25, mir-92a, mir-302b and mir-690) was

Table III. miRNAs differentially expressed between differentiated (day 10) and undifferentiated (day 0) P19 cells.

miRNA	P-value	log2 (day 10/day 0)
mmu-mir-1892	4.28E-10	3.91
mmu-mir-762	3.64E-09	3.91
mmu-mir-689	2.06E-08	3.25
mmu-mir-1224	2.70E-08	2.84
mmu-mir-2134	5.37E-08	3.22
mmu-mir-1894-3p	3.15E-07	1.98
mmu-mir-2132	4.00E-07	2.27
mmu-mir-2138	7.18E-07	2.32
mmu-mir-705	8.35E-07	3.13
mmu-mir-2133	1.63E-06	3.24
mmu-mir-2135	2.59E-06	4.17
mmu-mir-494	3.65E-06	2.09
mmu-mir-2141	9.83E-06	3.81
mmu-mir-671-5p	2.10E-05	3.09
mmu-mir-1956	2.17E-05	5.26
mmu-mir-2146	2.70E-05	4.24
mmu-mir-2145	2.75E-05	1.78
mmu-mir-714	5.18E-05	4.56
mmu-mir-680	1.14E-04	2.35
mmu-mir-2140	1.90E-04	1.56
mmu-mir-294	9.01E-04	1.09
mmu-mir-1	1.56E-03	0.63
mmu-mir-199a-3p	2.96E-03	5.90
mmu-mir-19b	4.50E-10	-3.99
mmu-mir-690	1.34E-08	-1.97
mmu-mir-720	8.38E-08	-2.64
mmu-mir-125a-5p	1.13E-07	-1.96
mmu-mir-805	1.27E-07	-1.99
mmu-mir-1937b	3.63E-06	-1.54
mmu-mir-106a	5.60E-06	-1.29
mmu-mir-25	7.01E-06	-1.52
mmu-mir-1937a	7.07E-06	-1.31
mmu-mir-21	1.06E-05	-1.79
mmu-mir-92b	1.26E-05	-1.49
mmu-mir-15b	2.67E-05	-1.35
mmu-mir-382	2.97E-05	-1.43
mmu-mir-16	4.25E-05	-2.09
mmu-mir-20a	5.03E-05	-1.55
mmu-mir-92a	5.34E-05	-1.95
mmu-mir-335-5p	6.23E-05	-0.94
mmu-mir-20b	1.00E-04	-1.25
mmu-mir-23b	1.11E-04	-1.21
mmu-mir-17	1.25E-04	-1.66
mmu-mir-125b-5p	1.51E-04	-1.30
mmu-mir- 302b	2.58E-04	-1.00
mmu-mir-292-5p	2.79E-04	-0.61
mmu-mir-302a	4.49E-04	-0.61
mmu-mir-379	9.52E-04	-1.28
mmu-mir-23a	2.72E-03	-0.52

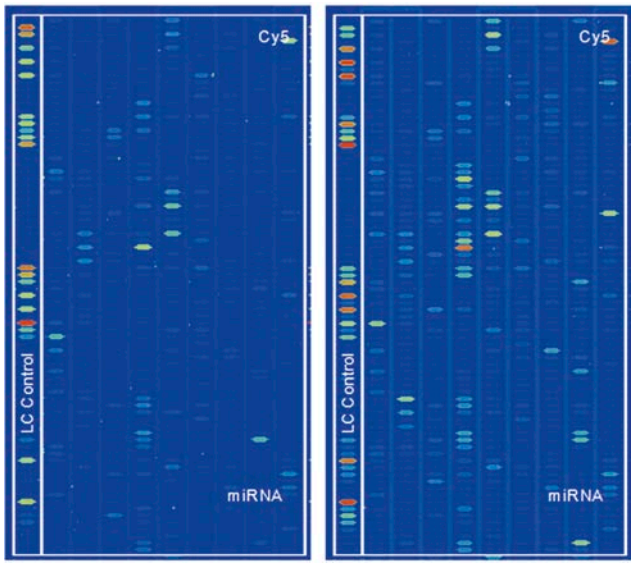

Figure 3. Representative images of chip regions. The miRNA profiles can be read directly from these Cy5 images. The images are displayed in pseudo colors to expand the visual dynamic range. As the signal intensity increases from 1 to 65,535 the corresponding color changes from blue to green, to yellow and then to red. Left panel, day 0; right panel, day 10.

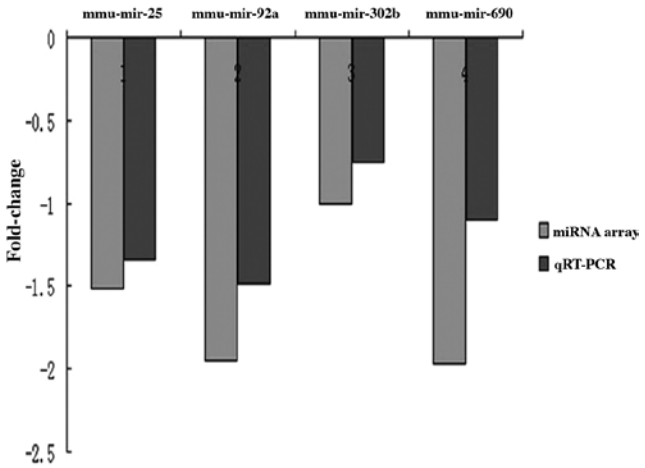

Figure 4. Comparison of miRNA expression measurements by microarray and real-time qRT-PCR. Triplicate assays were performed from each RNA sample. Data are normalized using U6 as an endogenous control for RNA input. Fold-changes for these miRNAs from array and real-time qRT-PCR are shown as the mean.

analyzed. The expression data obtained by real-time qRT-PCR analysis were comparable to the microarray data (Fig. 4).

Target prediction analysis. For this analysis, we selected the down-regulated miRNA (mmu-mir-19b) which demonstrated the highest degree of differential expression with the lowest P-value (most significant difference) in order to identify its potential gene targets. We thus obtained the mmu-mir-19b target genes wnt1, wnt3 and wnt7b.

Discussion

The heart is one of the first organs to function in the developing embryo. Although several key elements of the transcriptional

network that orchestrates vertebrate heart development have been successfully identified, little is known about the post-transcriptional control mechanisms of this process (26). In this study, we differentiated normal P19 cells into beating cardiac myocytes, and confirmed the differentiation by detecting cTnI expression. Forty-nine differentially expressed miRNAs were found in cardiac myocytes compared to normal P19 cells; 23 were up-regulated and 26 down-regulated. The microarray results were confirmed for 4 selected miRNAs genes (mir-25, mir-92a, mir-302b and mir-690) with real-time qRT-PCR. We also obtained the targets gene wnt1, wnt3 and wnt7b of mmu-mir-19b by target prediction analysis. Wnt signaling promotes the differentiation of cardiomyocytes (19,27). It is therefore possible that mir-19b plays an important role during the differentiation of P19 cells into cardiac myocytes through the wnt genes.

The precursor transcript derived from the mir-17-92 cluster contains six tandem stem-loop hairpin structures that ultimately yields six mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1 (28). Expression profiling studies have revealed the widespread overexpression of these miRNAs in diverse tumor subtypes including tumors from both hematopoietic malignancies and solid tumors such as those derived from the breast, colon, lung, pancreas, prostate, and stomach (7,29). Ventura *et al* documented that loss-of-function of the miR-17-92 cluster resulted in smaller embryos and immediate postnatal death of all animals. This was likely due to severely hypoplastic lungs and ventricular septal defects in the hearts of mice lacking miR-17-92 (30). In the present study, mir-17, mir-19b, mir-20a, mir-20b, mir-92a and mir-92b were down-regulated, and may therefore participate in maintaining P19 cell self-renewal and inhibiting cardiac differentiation. The mir-21 expression level is dysregulated in the heart and vasculature in cardiovascular diseases such as proliferative vascular disease, cardiac hypertrophy, heart failure and ischemic heart disease (31). Members of the mir-302 family are down-regulated rapidly in response to embryonic stem cell (ESC) differentiation (32). Lee *et al* also demonstrated that functional miR-302b is necessary to maintain stem cell self-renewal and inhibit the differentiation of human embryonic carcinoma cells (ECCs) (33), which is consistent with our results. The function of these miRNAs, and the mechanism by which they participate in heart development, requires further study.

While many of the miRNAs identified as differentially expressed in the present study have known disease correlations, others, such as mir-1892, mir-2132, and mir-2133, do not. The results of this study suggest that investigation into their function and mechanism could provide further information about the regulation of cardiomyocyte differentiation. Diagnostic use and therapeutic modulation of individual miRNAs or miRNA clusters in cardiovascular diseases may become a promising clinical solution.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (Grant No. 81070138), and the Natural Science Foundation of the Jiangsu Province, China (Grant No. BK2010582).

References

1. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ and Srivastava D: Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell 129: 303-317, 2007.
2. Hoffman JI and Kaplan S: The incidence of congenital heart disease. J Am Coll Cardiol 39: 1890-1900, 2002.
3. Xu P, Vernooy SY, Guo M and Hay BA: The *Drosophila* microRNA mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol 13: 790-795, 2003.
4. Cheng AM, Byrom MW, Shelton J and Ford LP: Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res 33: 1290-1297, 2005.
5. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M and Croce CM: miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 102: 13944-13949, 2005.
6. Dresios J, Aschrafi A, Owens GC, Vanderklish PW, Edelman GM and Mauro VP: Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. Proc Natl Acad Sci USA 102: 1865-1870, 2005.
7. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC and Croce CM: A microRNA expression signature of human solid tumors defnes cancer gene targets. Proc Natl Acad Sci USA 103: 2257-2261, 2006.
8. Calin GA, Garzon R, Cimmino A, Fabbri M and Croce CM: MicroRNAs and leukemias: how strong is the connection? Leuk Res 30: 653-655, 2006.
9. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR and Ruvkun G: The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. Nature 403: 901-906, 2000.
10. Calin GA and Croce CM: MicroRNA signatures in human cancers. Nat Rev Cancer 6: 857-866, 2006.
11. Garzon R, Pichiorri F, Palumbo T, Visentini M, Aqeilan R, Cimmino A, Wang H, Sun H, Volinia S, Alder H, Calin GA, Liu CG, Andreeff M and Croce CM: MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. Oncogene 26: 4148-4157, 2007.
12. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
13. Mack GS: MicroRNA gets down to business. Nat Biotechnol 25: 631-638, 2007.
14. Scalbert E and Bril A: Implication of microRNAs in the cardiovascular system. Curr Opin Pharmacol 8: 181-188, 2008.
15. Hatfield S and Ruohola-Baker H: microRNA and stem cell function. Cell Tissue Res 331: 57-66, 2008.
16. Zhang W, Dahlberg JE and Tam W: MicroRNAs in tumorigenesis: a primer. Am J Pathol 171: 728-738, 2007.
17. Skerjanc IS: Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. Trends Cardiovasc Med 9: 139-143, 1999.
18. van der Heyden MA and Defize LH: Twenty-one years of P19 cells: what an embryonal carcinoma cell line taught us about cardiomyocyte differentiation. Cardiovasc Res 58: 292-302, 2003.
19. Han SP, Pan Y, Peng YZ, Gu XQ, Chen RH and Guo XR: Folbp1 promotes embryonic myocardial cell proliferation and apoptosis through the WNT signal transduction pathway. Int J Mol Med 23: 321-330, 2009.
20. Hu DL, Chen FK, Liu YQ, Shen YH, Yang R, Kong XQ, Cao KJ, Gu HT and Qian LM: *GATA*-4 promotes the differentiation of P19 cells into cardiac myocytes. Int J Mol Med 26: 365-372, 2010.
21. van der Heyden MA, van Kempen MJ, Tsuji Y, Rook MB, Jongsma HJ and Opthof T: P19 embryonal carcinoma cells: a suitable model system for cardiac electrophysiological differentiation at the molecular and functional level. Cardiovasc Res 58: 410-422, 2003.
22. Kaczocha M, Glaser ST and Deutsch DG: Identification of intracellular carriers for the endocannabinoid anandamide. Proc Natl Acad Sci USA 106: 6375-6380, 2009.
23. Gao X, Gulari E and Zhou X: *In situ* synthesis of oligonucleotide microarrays. Biopolymers 73: 579-596, 2004.

24. Zhu Q, Hong A, Sheng N, Zhang X, Matejko A, Jun KY, Srivannavit O, Gulari E, Gao X and Zhou X: microParaflo biochip for nucleic acid and protein analysis. Methods Mol Biol 382: 287-312, 2007.
25. Bolstad BM, Irizarry RA, Astrandand M and Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185-193, 2003.
26. Meder B, Katus HA and Rottbauer W: Right into the heart of microRNA-133a. Genes Dev 22: 3227-3231, 2008.
27. Kwon C, Arnold J, Hsiao EC, Taketo MM, Conklin BR and Srivastava D: Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. Proc Natl Acad Sci USA 104: 10894-10899, 2007.
28. Tanzer A and Stadler PF: Molecular evolution of a microRNA cluster. J Mol Biol 339: 327-335, 2004.
29. Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, Iliopoulos D, Pilozzi E, Liu CG, Negrini M, Cavazzini L, Volinia S, Alder H, Ruco LP, Baldassarre G, Croce CM and Vecchione A: E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell 13: 272-286, 2008.
30. Ventura A, Young AG, Winslow MM, Linault L, Meissner A, Erkeland SJ, Newman J, Bronson RT, Crowley D, Stone JR, Jaenisch R, Sharp PA and Jacks T: Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell 132: 875-886, 2008.
31. Cheng Y and Zhang C: MicroRNA-21 in cardiovascular disease. J Cardiovasc Transl Res 3: 251-255, 2010.
32. Stadler B, Ivanovska I, Mehta K, Song S, Nelson A, Tan Y, Mathieu J, Darby C, Blau CA, Ware C, Peters G, Miller DG, Shen L, Cleary MA and Ruohola-Baker H: Characterization of microRNAs involved in embryonic stem cell states. Stem Cells Dev 19: 935-950, 2010.
33. Lee NS, Kim JS, Cho WJ, Lee MR, Steiner R, Gompers A, Ling D, Zhang J, Strom P, Behlke M, Moon SH, Salvaterra PM, Jove R and Kim KS: miR-302b maintains 'stemness' of human embryonal carcinoma cells by post-transcriptional regulation of Cyclin D2 expression. Biochem Biophys Res Commun 377: 434-440, 2008.