Biochemical and Biophysical Research Communications 428 (2012) 191–196

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of novel microRNAs negatively regulating cardiac hypertrophy

Moon Hee Jeong, Jong Sub Lee, Do Han Kim, Woo Jin Park [1,*], Dong Kwon Yang [1,*]

College of Life Sciences, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea

ARTICLE INFO

Article history:
Received 25 September 2012
Available online 12 October 2012

Keywords:
Cardiac hypertrophy
Regression
MicroRNA
Cardiomyocytes
Transverse aortic constriction

ABSTRACT

MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules that suppress gene expression via degradation or translational inhibition of their target genes. Many miRNAs are associated with cardiac hypertrophy and heart failure. In this study, we pursued to identify miRNAs that negatively regulate cardiac hypertrophy by utilizing a surgical model for regression of cardiac hypertrophy. Microarray analysis revealed that 15 miRNAs out of the 696 miRNAs tested were specifically up-regulated during the regression period. Among these regression-specific miRNAs, nine microRNAs, which have not been previously reported, were further tested for their effects on phenylephrine (PE)-treated neonatal cardiomyocytes. Consequently, five miRNAs (miR-101b, 142-3p, 181d, 24-2*, and 450a) completely abrogated PE-induced hypertrophy as determined by measurements of cell size and fetal gene expression. Conversely, antagomers of these miRNAs exacerbated the PE-induced hypertrophy. Collectively, these findings suggest that the five miRNAs newly identified by using our cardiac hypertrophy-regression surgical model negatively regulate cardiac hypertrophy and could be used as potential therapeutic targets for the treatment of heart diseases.

1. Introduction

Cardiac hypertrophy is a remodeling of myocardium marked by the enlargement of cardiac myocytes, which occurs in response to various pathological stimuli including hypertension, valvular disease, and myocardial infarction [1–3]. It is considered a compensatory response in its initial stages. However, it often leads to progression to heart failure when hypertrophic stimuli persist. Therefore, cardiac hypertrophy is an independent and major risk factor for cardiovascular morbidity and mortality [4].

MicroRNAs (miRNAs) are ~22 nucleotide small RNAs that negatively regulate gene expression in post-transcriptional gene regulation by destabilizing mRNAs or inhibiting their translations [5]. Recently, a number of miRNAs have been implicated in various cardiovascular diseases, including cardiac hypertrophy, heart failure and cardiac arrhythmias [6]. In addition, miRNA expression profiling studies have shown that many miRNAs are differentially expressed and play crucial roles in the process of cardiac hypertrophy with either pro- or anti-hypertrophic activity [7,8]. Several miRNAs promote cardiac hypertrophy by inhibiting the expression of target genes involved in cell survival, differentiation, and anti-hypertrophic signaling pathways [9]. A few miRNAs that negatively regulate cardiac hypertrophy have also been identified, although the mechanism underlying their anti-hypertrophic effects has yet to be fully elucidated.

Using a surgical model for regression of cardiac hypertrophy, we have previously shown that some genes specifically induced during the regression period are anti-hypertrophic [10]. In this study, we utilized the same surgical model and isolated miRNAs that were induced during the regression period. Combined with functional tests in neonatal cardiomyocytes, we finally identified five novel miRNAs that negatively regulate cardiac hypertrophy. Further studies with these novel miRNAs will provide new insights into the regulatory mechanism of cardiac hypertrophy and open the prospect of using these novel miRNAs as therapeutic targets for the treatment of cardiac diseases.

2. Materials and methods

2.1. Surgical procedures

All animal experiments in this study were performed with the approval of the Animal Care Committee of Gwangju Institute of Science and Technology. Pressure overload induced hypertrophy in the mouse heart was performed. Briefly, male mice of 8–10 weeks of age (20–25 g) were anesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (2.5 mg/kg). A longitudinal incision of 2–3 mm was made in the proximal sternum to allow visualization of the aortic arch, while the mice were constantly ventilated (Harvard Apparatus). The

* Corresponding authors. Address: College of Life Sciences, Gwangju Institute of Science and Technology, 1 Oryong-dong, Buk-gu, Gwangju 500-712, Republic of Korea. Fax: +82 62 715 2484 (W.J. Park), fax: +82 62 715 2484 (D.K. Yang).
E-mail addresses: woojinpark@me.com (W.J. Park), dode@gist.ac.kr (D.K. Yang).
[1] These authors are co-corresponding authors.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.10.040

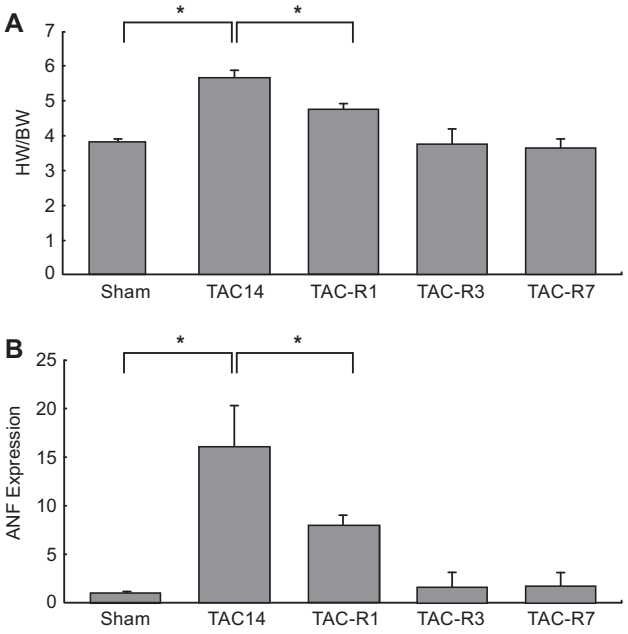

Fig. 1. TAC-induced cardiac hypertrophy and regression of the established cardiac hypertrophy. (A) Assessment of heart weight/body weight (HW/BW) (mean ± SD, $n = 4–6$ for each group). (B) Quantitative RT-PCR analysis for ANF transcript levels in hearts at each point (Sham, TAC14, TAC-R1, TAC-R3 and TAC-R7). The expression was presented as fold induction over sham. $^{*}P < 0.05$ *vs* sham group. TAC, transverse aortic constriction; TAC14, 14 days after TAC; TAC-R1, R3 and R7; 14 days after TAC with 1 day, 3 days and 7 days of constriction relief.

transverse aorta was then banded (7-0 silk) with an overlaying blunted 27-gauge needle, and then the needle was quickly removed to create a defined constriction. Sham operations were also performed in which the transverse aorta was exposed but not banded. To unload the hypertrophied hearts, the constriction was relieved by untying the silk ligature 2 weeks after the initial banding surgery. Before and after relieving the constriction, we monitored the pressure in the right carotid artery to confirm the restoration of normal aortic blood flow.

2.2. cDNA microarray

Total RNA was prepared from 4 to 5 left ventricles for each experiment (Trizol) and subjected to hybridization to the Agilent Mouse miRNA Microarray, which contains about 696 miRNAs according to the manufacturer's recommendations by E-biogen (Seoul, Korea). The processed slides were scanned with an Agilent's DNA microarray scanner and analyzed with the Feature Extraction Software (Agilent). Reproducibility of the obtained data was assessed by scatter-plot analysis of normalized probe signals. The data set with the lowest correlation coefficient among the data sets at each point was discarded, and the remaining data sets were subjected to further analysis.

2.3. Cell culture

Neonatal rat cardiomyocytes were obtained from 1 to 2 days old Sprague–Dawley rats as described previously [11]. In brief, ventricular tissue was enzymatically dissociated, and the resulting cell suspension was enriched for cardiomyocytes using Percoll (Amersham) step gradients. Isolated cardiomyocytes were plated onto either collagen-coated culture dishes or coverslips and then cultured in cardiomyocyte culture medium (DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 μM 5-bromodeoxyuridine; GIBCO-BRL).

2.4. Transfection of miRNA and hypertrophic stimulation

Rat miR 101b, 142-3p, 181d, 24-2*, 450a and rat anti-miR 101b, 142-3p, 181d, 24-2*, 450a and miR negative control were purchased from Dharmacon. Neonatal rat cardiomyocytes were cultured in serum-free medium for at least 24 h and then each miRNA (100 nM) were transfected into cardiomyocytes using lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). After 48 h, to induce hypertrophy, cardiomyocytes were treated with 100 μM Phenylephrine (PE) for 24 h. The sequences of miRNAs mimics and anti-miR are shown in Supplementary Table 1.

2.5. Immunostaining and cell size measurement

After experimental treatment, neonatal rat cardiomyocytes grown on collagen-coated cover slips were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked by incubation in 5% BSA solution for 1 h at room temperature. The cells were incubated with anti-α-actinin antibody (1:200; Sigma) at 4 °C overnight, and as a secondary antibody, Alexa 488-coupled anti-mouse antibody (1:200; Invitrogen) was added and incubated for 1 h at room temperature. Immunofluorescence was analyzed under a microscope equipped with 40× objective lens and filters for epifluorescence (Olympus). Cell surface areas were measured using NIH image software.

2.6. Quantitative real-time PCR

Total RNA was isolated from neonatal rat cardiomyocytes or mouse cardiac ventricular tissues with TRI reagent (Sigma). To assess cardiac hypertrophic marker gene expression level, reverse-transcriptase reactions were performed using ImProm II reverse-transcriptase (Promega) with oligo-dT priming. Quantitative real-time PCR (qRT-PCR) was performed using a TaKaRa Thermal Cycler TP 815 with SYBR Green (Takara) as fluorescent dyes. The quantification of miRNAs was conducted by qRT-PCR using GenoExplorer™ miRNA First-strand cDNA core kit (GenoSensor) and GenoExplorer™ miRNA qPCR primer set (GenoSensor). The primers used were as follows: ANF Forward: 5′-ACCTGCTAGACCACCTAGAGG-3′, Reverse: 5′-GCTGTTATCTTCCGTACCGG-3′; β-MHC forward: 5′-CAGACATAGAGACCTACCTTC-3′, Reverse: 5′-CAGCATGTC TAGAAGCTCAGG-3′; GAPDH forward primer: 5′-CTCTACCCACGG CAAGTTC-3′, Reverse: 5′-GCCAGTAGACTCCACGACATA-3′.

2.7. Statistics

All data are reported as mean ± SD. Statistical significance was analyzed by Student's *t* test or one-way ANOVA with a Bonferroni post hoc analysis (Statview 5.0, SAS). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Regression of cardiac hypertrophy

The transverse aortic constriction (TAC) was applied for 14 days to induce cardiac hypertrophy, and then the constriction was relieved by surgically removing the silk ligature to initiate the regression of established cardiac hypertrophy. For the analysis, we selected five time points: Sham-operated (Sham), 14 days after TAC (TAC14), and 14 days after TAC with 1, 3 and 7 days of constriction relief (TAC-R1, TAC-R3 and TAC-R7, relatively). Development of cardiac hypertrophy and regression of the established cardiac hypertrophy were confirmed by measuring the ratio of heart weight to body weight (16% decrease in TAC-R1 hearts

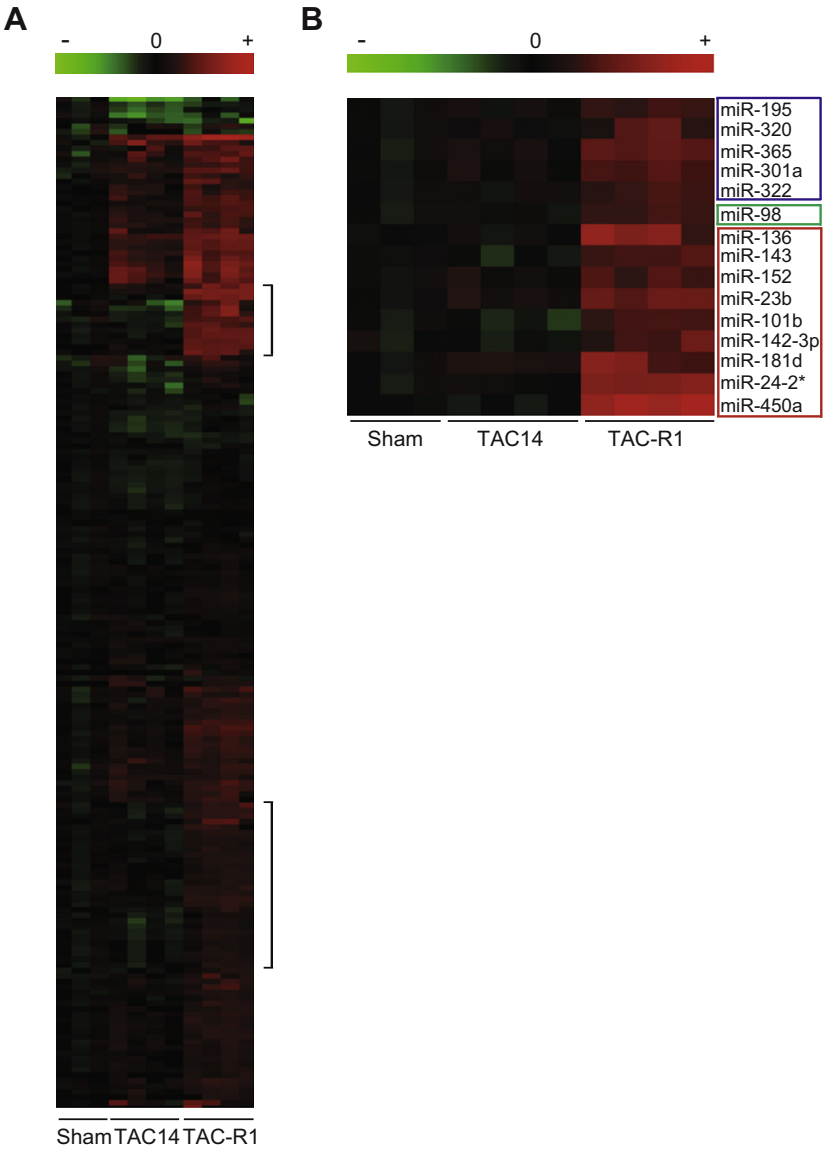

Fig. 2. Clustering of miRNA expression. (A) Clustered miRNA expression patterns of 696 miRNAs are shown graphically. Each row represents a different miRNA, and each column displays miRNA expressions at each point (Sham, TAC14, TAC-R1). Data values displayed as red and green represent elevated and reduced expression, respectively. (B) The miRNAs that were specifically ($P < 0.05$) up-regulated in the regression of cardiac hypertrophy (TAC-R1 group). The blue box indicates cardiac hypertrophy or stress inducers, and the green box indicates negative regulators. The red box indicates the miRNAs that have not previously identified. TAC, transverse aortic constriction; TAC-R1, 14 days after TAC with 1 day of constriction relief. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

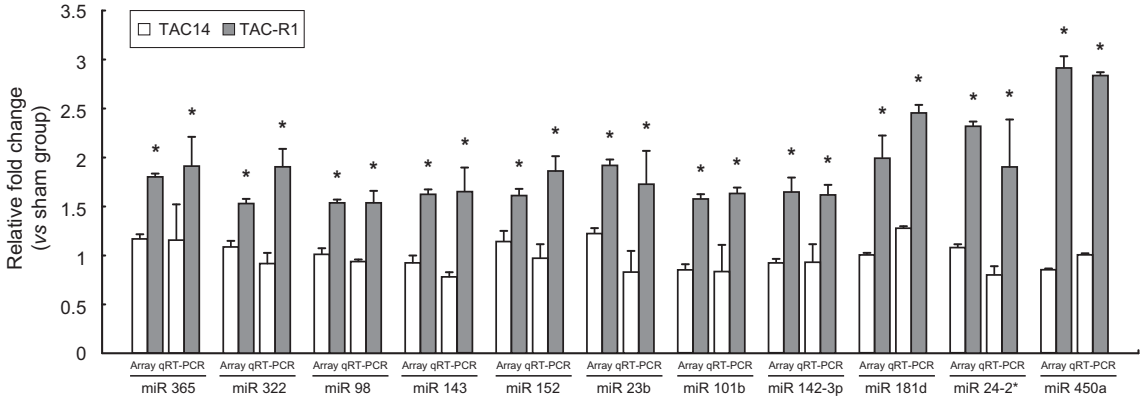

Fig. 3. Quantitative real-time PCR (qRT-PCR) of selected regression-specific miRNAs. qRT-PCR was performed in triplicate with 4–5 independent heart samples at each point (Sham, TAC14 and TAC-R1). The expression was presented as fold induction over sham. $^{*}P < 0.05$ *vs* sham group. TAC, transverse aortic constriction; TAC14, 14 days after TAC; TAC-R1; 14 days after TAC with 1 day of constriction relief.

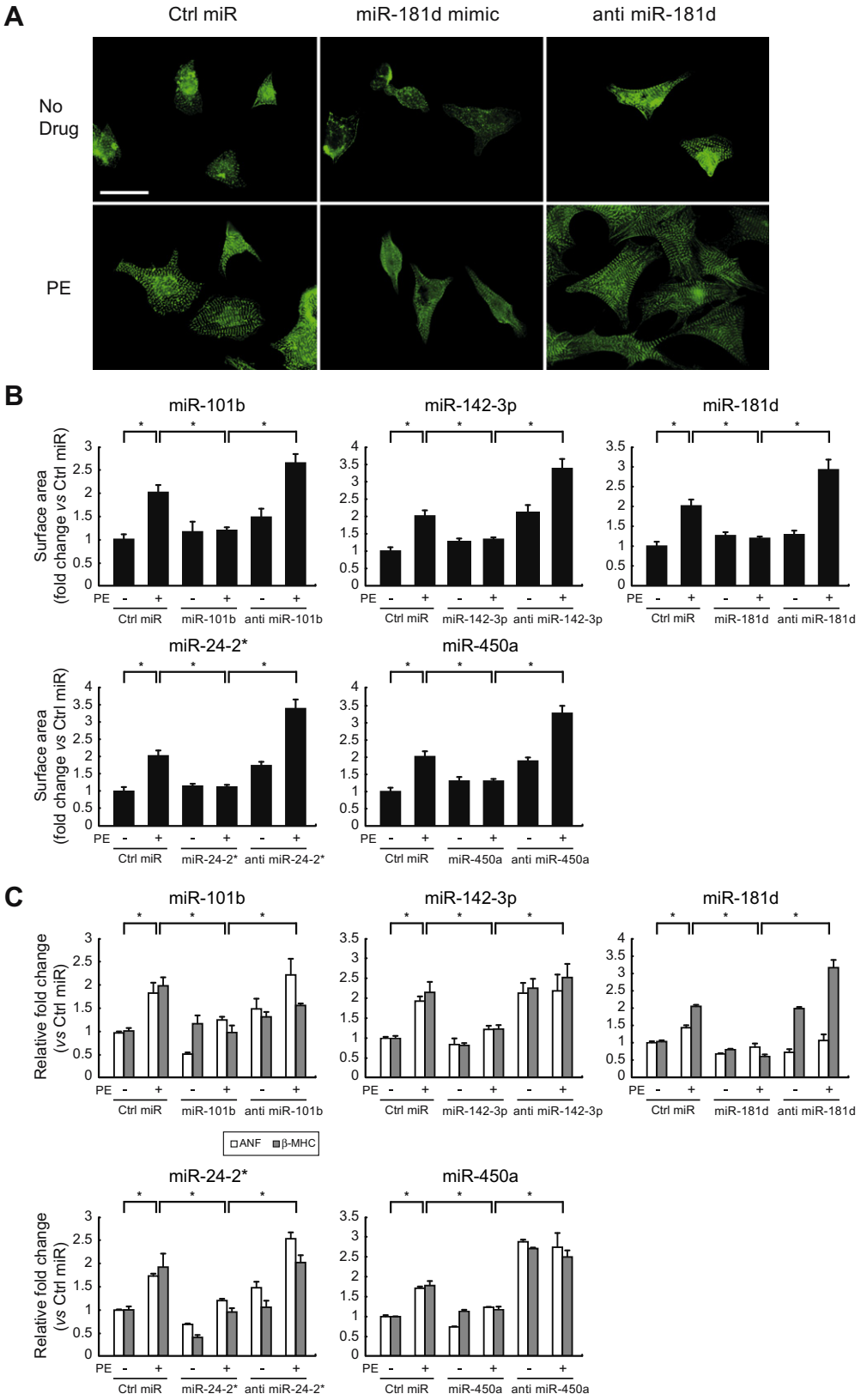

Fig. 4. Regressioin-specific miRNAs (miR-101b, 142-3p, 181d, 24-2* and 450a) inhibit the hypertrophic response in phenylnephrine (PE)-treated neonatal cardiomyocytes. (A) Representative photograph of cardiomyocytes transfected with miR-181d mimic or anti-miR-181d after PE treatment. Sarcomeric organization of the cardiomyocytes was visualized by staining with anti-actinin antibody and occurred in response to PE treatment. The response was completely blocked by transfecting with either one of the five regression-specific miRNAs. Conversely, antigomers of these miRNAs exacerbated cardiac hypertrophy after PE treatment. (B) Cell surface areas of the cardiomyocytes were measured using Image J software (n = 100). Scale bars, 50 μm. (C) Quantitative RT-PCR analysis for ANF and β-MHC expression in PE-treated rat cardiomyocytes treated with miRNA mimics or miRNA antagomers. Data are expressed as fold changes ±SD *vs* control-miR transfection. $^{*}P < 0.05$. Ctrl-miR, control-miR; PE, phenylephrine.

compared with TAC14 hearts; Fig. 1A) and atrial natriuretic factor (ANF) expression levels (43.4% decrease compared with TAC14 hearts; Fig. 1B). At TAC-R3 and TAC-R7, cardiac hypertrophy appeared to have completely regressed.

3.2. Identification of miRNAs specific to regression of cardiac hypertrophy

Total RNA was prepared and hybridized to Agilent microarrays containing 696 miRNAs. Average values of the miRNA expression levels at each time point were clustered and illustrated graphically (Fig. 2A). Hierarchical clustering revealed distinct clusters of miRNAs which expression was up-regulated only in the regressing hearts (black lines). Among the miRNAs belonging to these clusters, 15 miRNAs showed statistically significant ($P < 0.05$) up-regulation in the regressing hearts but not in hypertrophied hearts (Fig. 2B). We assumed that these miRNAs might be involved in the development or regression of cardiac hypertrophy. Among these miRNAs, miR-195, miR-320, miR-365, miR-301a, and miR-322 were previously reported to be either pro-hypertrophic regulators or stress inducers, and miR-98 was previously shown to inhibit cardiac hypertrophy in angiotensin II-treated cardiomyocytes. The functions of the remaining nine miRNAs (miR-136, miR-143, miR-152, miR-23b, miR-101b, miR-142-3p, miR-181d, miR-24-2*, and miR-450a) have not been reported previously.

3.3. Confirmation of regression-specific miRNAs expression changes

To confirm the validity of miRNA microarray data, we performed quantitative RT-PCR (qRT-PCR) with eleven representative regression-specific miRNAs. The expression changes assayed by qRT-PCR were generally similar to the changes in expression as determinded by miRNA microarrays (Fig. 3).

3.4. miR-101b, miR-42-3p, miR-181d, miR-24-2*, and miR-450a as negative regulators of cardiac hypertrophy

To elucidate the functions of the nine miRNAs, mimics of these miRNAs were transfected into phenylnephrine (PE)-treated primary neonatal rat cardiomyocytes. Untransfected or transfected cardiomyocytes were cultured for 48 h and were then further stimulated with PE for 24 h. The hypertrophic response of cardiomyocytes is characterized by an increased cell size and a pronounced sarcomeric rearrangement that can be detected by immunostaining with α-actinin antibody. While mimics for miR-136, miR-143, miR-152, and miR-23b had obscure or no anti- or pro-hypertrophic effects (data not shown), mimics for miR-101b, miR-142-3p, miR-181d, miR-24-2*, and miR-450a significantly inhibited PE-induced cardiomyocyte hypertrophy. In addition, while antagomers for miR-136, miR-143, miR-152, and miR-23b had no effects (data not shown), antagomers for miR-101b, miR-142-3p, miR-181d, miR-24-2*, and miR-450a significantly exacerbated PE-induced hypertrophic responses. A set of representative experiments for miR-450a is shown in Fig. 4A and measurements for cell surface areas for the five miRNAs are shown in Fig. 4B.

Quantitative RT-PCR (qRT-PCR) was carried out to confirm the anti-hypertrophic effects of the miRNAs. The PE-induced increase in ANF and β-MHC expression was significantly inhibited by treatment with mimics and exacerbated by treatment with antagomers of five miRNAs (Fig. 4C). Collectively, these results suggested that the five regression period-specific miRNAs are negative regulators of cardiac hypertrophy.

4. Discussion

Cardiac hypertrophy is a major risk factor for heart failure [12,13]. There have been intensive efforts to identify negative as well as positive regulators of cardiac hypertrophy and elucidate their underlying anti-hypertrophic mechanisms [14]. Up to now, several miRNAs including miR-133 and miR-1 have been reported to negatively regulate cardiac hypertrophy [15]. The expression of these miRNAs decreased in mouse and human models of cardiac hypertrophy, and their inhibitory functions in cardiac hypertrophy was elucidated by performing '*gain and loss*' functional studies both *in vitro* and *in vivo*.

We have previously identified cardiac hypertrophy regression specific genes by utilizing a surgical model for regression of cardiac hypertrophy [10]. We hypothesized that the anti-hypertrophic signaling pathways were activated against cardiac hypertrophy when cardiac hypertrophy was regressed. Consequently, some of the genes specifically up-regulated during the regression of cardiac hypertrophy might be involved in anti-hypertrophic signaling pathways. Indeed, Eya2, a regression-specific gene, was shown to be a negative regulator of cardiac hypertrophy [16]. In this study, we utilized the surgical model for regression of cardiac hypertrophy to isolate miRNAs that are specifically up-regulated during the regression period. Among the 696 miRNAs tested, we found 15 miRNAs that showed statistically meaningful up-regulations during the regression period. Among these, six miRNAs (miR-195, miR-301a, miR-320, miR-322, miR-365, and miR-98) have previously been associated with various cellular processes in cardiomyocytes. For example, miR-195 and miR-365 promoted apoptosis by inhibiting Sirt1 expression in cardiomyocytes and Bcl-2 expression in the HUVEC cell line, respectively [17,18]. miR-320 regulated cardiac ischemia-reperfusion injury by targeting heat-shock protein 20. miR-301a and 322 are stress-inducible miRNAs that inhibit the expression of their target genes, NF-κB-repressing factor (Nkrf) and Cdc25A, respectively [19,20]. miR-98 negatively regulates angiotensin II-induced cardiac hypertrophy via down-regulation of cyclin D2 expression [21].

Among the nine new miRNAs identified in this study, five miRNAs (miR-101b, miR-142-3p, miRNA-181d, miRNA-24-2*, and miR-450a) negatively regulated cardiac hypertrophy. To further elucidate the molecular mechanism underlying the anti-hypertrophic effects of these miRNAs, their target genes should be identified. It would be also important to determine whether these miRNAs can ameliorate various cardiac diseases (e.g. heart failure, cardiac infarction and ischemia and reperfusion injury) *in vivo*.

In conclusion, we identified five novel negative regulators of cardiac hypertrophy by using our established surgical model for regression of cardiac hypertrophy. Elucidating how they counteract cardiac hypertrophy should valuable insights into the inhibitory mechanisms underlying cardiac hypertrophy. These novel miRNAs may provide diagnostic and therapeutic targets for cardiovascular disorders.

Acknowledgments

D.K.Y. was supported by a grant (2010-0010754) from the Basic Science Research Program of the Korean Ministry of Education, Science and Technology. W.J.P. was supported by a grant (M6-0605-00-0001) from the Global Research Laboratory Program of the Korean Ministry of Education, Science and Technology, a grant (A101749) from the Korean Health Technology R&D Project of Korean Ministry of Health, Welfare & Family Affairs, and a grant from the Systems Biology Infrastructure Establishment Program of GIST.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.040.

References

[1] J. Heineke, J.D. Molkentin, Regulation of cardiac hypertrophy by intracellular signalling pathways, Nature Reviews Molecular Cell Biology 7 (2006) 589–600.
[2] G.W. Dorn 2nd, J. Robbins, P.H. Sugden, Phenotyping hypertrophy: eschew obfuscation, Circulation Research 92 (2003) 1171–1175.
[3] J.J. Hunter, K.R. Chien, Signaling pathways for cardiac hypertrophy and failure, The New England Journal of Medicine 341 (1999) 1276–1283.
[4] A.M. Katz, Cardiomyopathy of overload. A major determinant of prognosis in congestive heart failure, The New England Journal of Medicine 322 (1990) 100–110.
[5] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[6] D. Sayed, C. Hong, I.Y. Chen, J. Lypowy, M. Abdellatif, MicroRNAs play an essential role in the development of cardiac hypertrophy, Circulation Research 100 (2007) 416–424.
[7] E. van Rooij, L.B. Sutherland, N. Liu, A.H. Williams, J. McAnally, R.D. Gerard, J.A. Richardson, E.N. Olson, A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure, Proceedings of the National Academy of Sciences of the United States of America 103 (2006) 18255–18260.
[8] C. Jentzsch, S. Leierseder, X. Loyer, I. Flohrschutz, Y. Sassi, D. Hartmann, T. Thum, B. Laggerbauer, S. Engelhardt, A phenotypic screen to identify hypertrophy-modulating microRNAs in primary cardiomyocytes, Journal of Molecular and Cellular Cardiology 52 (2012) 13–20.
[9] E.M. Small, R.J. Frost, E.N. Olson, MicroRNAs add a new dimension to cardiovascular disease, Circulation 121 (2010) 1022–1032.
[10] D.K. Yang, B.Y. Choi, Y.H. Lee, Y.G. Kim, M.C. Cho, S.E. Hong, H. Kim do, R.J. Hajjar, W.J. Park, Gene profiling during regression of pressure overload-induced cardiac hypertrophy, Physiological Genomics 30 (2007) 1–7.
[11] J. Sadoshima, L. Jahn, T. Takahashi, T.J. Kulik, S. Izumo, Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy, The Journal of Biological Chemistry 267 (1992) 10551–10560.
[12] D. Levy, R.J. Garrison, D.D. Savage, W.B. Kannel, W.P. Castelli, Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study, The New England Journal of Medicine 322 (1990) 1561–1566.
[13] N. Frey, E.N. Olson, Cardiac hypertrophy: the good, the bad, and the ugly, Annual Review of Physiology 65 (2003) 45–79.
[14] S.E. Hardt, J. Sadoshima, Negative regulators of cardiac hypertrophy, Cardiovascular Research 63 (2004) 500–509.
[15] M.M. Gladka, P.A. da Costa Martins, L.J. De Windt, Small changes can make a big difference – microRNA regulation of cardiac hypertrophy, Journal of Molecular and Cellular Cardiology 52 (2012) 74–82.
[16] S.H. Lee, D.K. Yang, B.Y. Choi, Y.H. Lee, S.Y. Kim, D. Jeong, R.J. Hajjar, W.J. Park, The transcription factor Eya2 prevents pressure overload-induced adverse cardiac remodeling, Journal of Molecular and Cellular Cardiology 46 (2009) 596–605.
[17] H. Zhu, Y. Yang, Y. Wang, J. Li, P.W. Schiller, T. Peng, MicroRNA-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating Sirt1, Cardiovascular Research 92 (2011) 75–84.
[18] B. Qin, B. Xiao, D. Liang, J. Xia, Y. Li, H. Yang, MicroRNAs expression in ox-LDL treated HUVECs: MiR-365 modulates apoptosis and Bcl-2 expression, Biochemical and Biophysical Research Communications 410 (2011) 127–133.
[19] Z. Lu, Y. Li, A. Takwi, B. Li, J. Zhang, D.J. Conklin, K.H. Young, R. Martin, miR-301a as an NF-kappaB activator in pancreatic cancer cells, The EMBO Journal 30 (2011) 57–67.
[20] S. Sarkar, B.K. Dey, A. Dutta, MiR-322/424 and -503 are induced during muscle differentiation and promote cell cycle quiescence and differentiation by down-regulation of Cdc25A, Molecular Biology of the Cell 21 (2010) 2138–2149.
[21] Y. Yang, T. Ago, P. Zhai, M. Abdellatif, J. Sadoshima, Thioredoxin 1 negatively regulates angiotensin II-induced cardiac hypertrophy through upregulation of miR-98/let-7, Circulation Research 108 (2011) 305–313.