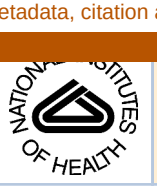

Published in final edited form as:

J Mol Cell Cardiol. 2007 June ; 42(6): 1137–1141.

Expression of microRNAs is dynamically regulated during cardiomyocyte hypertrophy

Mariko Tatsuguchi[1,2], **Hee Young Seok**[1,2], **Thomas E. Callis**[1,2], **J. Michael Thomson**[2], **Jian-Fu Chen**[1,2], **Martin Newman**[2], **Mauricio Rojas**[1], **Scott M. Hammond**[2], and **Da-Zhi Wang**[1,2,*]

1*Carolina Cardiovascular Biology Center, University of North Carolina, Chapel Hill, NC 27599*

2*Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC 27599*

Abstract

MicroRNAs (miRNAs) are a recently discovered class of ~22-nucleotide regulatory RNAs that post-transcriptionally regulate gene expression. We have recently demonstrated that muscle-specific miRNAs miR-1 and -133 play an important role in modulating muscle proliferation and differentiation. Here, we investigate the involvement of miRNAs in cardiac hypertrophy. We analyzed the global expression of miRNAs in agonist-induced hypertrophic cardiomyocytes as well as in pressure overload-induced hypertrophic hearts and found the miRNA expression profile altered in those hypertrophic conditions. We further show that inhibition of endogenous miR-21 or -18b augments hypertrophic growth. Conversely, introduction of functional miR-21 or -18b into cardiomyocytes represses myocyte hypertrophy. Together, our studies point to miRNAs as critical regulators of cardiac hypertrophy.

INTRODUCTION

microRNAs (miRNAs) are a class of short, non-coding RNA molecules that have recently emerged as important regulators of gene expression [1]. Many miRNAs are highly evolutionarily conserved and more than 400 miRNAs are known to exist in humans alone [1]. miRNAs negatively regulate target gene expression through miRNA complementarity to target sequence(s) within the 3' untranslated region of target mRNAs through translational repression or by degradation of bound RNA [1-3]. Recently, we and others have demonstrated that a subset of muscle-specific miRNAs, miR-1 and miR-133 in particular, play important roles in muscle cell proliferation and differentiation [4,5]. Given that miRNAs participate in such fundamental processes, we investigated the potential role for miRNAs in regulating the well-documented changes in gene expression that occur during cardiac hypertrophy.

In this report, we show that the expression of a subset of miRNAs is altered in hypertrophic cardiomyocytes. Interestingly, we found more miRNAs that are up-regulated than down-regulated in response to cardiac hypertrophy. We have used both overexpression and knockdown approaches to demonstrate that miR-18b and miR-21 repress hypertrophy in neonatal rat cardiomyocyte *in vitro*.

*Correspondence: E-mail: dawang@med.unc.edu, Phone: 919-843-4590

Conflict of interest statement: No conflicts declared.

MATERIALS AND METHODS

Cardiomyocyte tissue culture and transfection

Preparation of neonatal rat cardiomyocytes was as described [6,7]. LNA oligonucleotides and miRNA duplexes were transfected using Lipofectamine. Hypertrophic stimuli used: PE (100 μM), LIF (1,000 units/ml) and fetal bovine serum (FBS) 10%.

Analysis of microRNA expression by microarray

Total RNA was isolated from rat neonatal cardiomyocytes and mouse heart tissue (Trizol). Microarray production and hybridization methods have been described [8]. Five micrograms of labeled RNA from each treatment were hybridized to a microRNA microarray with 560 mouse and human miRNAs and analyzed using GenePix Pro (Axon Instruments). Median pixel intensity values were background subtracted and log-transformed (base 2). Significant Analysis of Microarray (SAM, Stanford University) was used to determine statistically significant changes in miRNA expression between treatments (q-value threshold < 0.05).

microRNA Northern Blot, RT-PCR analysis and immunocytochemistry

microRNA Northern Blot analyses were as described [4]. Semi-quantitative RT-PCR and immunocytochemical analyses were essentially as described [7]. For cell size measurement, ~200 cardiomyocytes stained with anti-α-actinin antibody from each treatment were randomly chosen for surface area measurement using a computerized morphometric system (ImageJ). Data were presented as average ± SEM. Student's t test was used for statistical analysis. A PCR method to detect mature miRNAs was adapted with minor modification [9]. Total RNA was DNAse I treated, phenol-chloroform extracted, precipitated and dissolved in DEPC water. RNA was polyadenylated, then reversed-transcribed using an oligo(dT) adapter primer. Resulting cDNA was used in a PCR reaction with oligonucleotide primers complementary to the oligo(dT) adapter primer and to miR-18b. PCR products were resolved on a 15% acrylamide gel with a 10 bp DNA ladder.

Pressure-overload hypertrophy models

Male C57BL6 mice (6-8 wk old) were subjected to pressure overload by thoracic aortic banding (TAB) [10]. Mice were sacrificed after 2 wk or 4 wk banding and hearts harvested for RNA extraction.

RESULTS AND DISCUSSION

Global analysis of miRNA expression during cardiac hypertrophy

To better understand the role of miRNAs in cardiac hypertrophy, we sought to identify miRNAs involved in cardiac hypertrophy using a custom miRNA microarray screening approach [4, 8]. Using this sensitive and quantitative high-throughput technology, we profiled global miRNA expression in phenylephrine (PE)-treated neonatal cardiomyocytes (Figure 1A) and in mouse hearts subjected to pressure overload by thoracic aortic banding (TAB) (Figure 1B and F), and found the expression signatures of several miRNAs were altered during cardiac hypertrophy.

Northern blot analysis for several miRNAs confirmed the results from the miRNA microarray screening (Figure 1C and D). The expression of miR-21, -23a, and 125b is increased in agonist-induced hypertrophic cardiomyocytes and in the TAB-induced hypertrophic hearts. Whereas the expression of miR-25 and -29a is very low in neonatal cardiomyocytes and appears unchanged during hypertrophy *in vitro* (Figure 1C), their expression is drastically up regulated in TAB-induced hypertrophic hearts (Figure 1D). Interestingly, miR-18b is among the most

highly induced miRNAs in both *in vitro* and *in vivo* hypertrophy models (Figure 1), however, we were unable to confirm its expression by Northern analysis, due to its low expression in cardiomyocytes (data not shown). We applied a technique designed to detect expression of a mature miRNA by PCR [9], which confirmed increased miR-18b expression in response to hypertrophic stimuli (Figure 1E).

Inhibition of cardiomyocyte hypertrophy by miR-21 and miR-18b

To explore the potential involvement of miRNAs in the regulation of cardiomyocyte hypertrophy, we specifically inhibited miR-21 and -18b in neonatal rat cardiomyocytes using locked nucleic acid (LNA)-modified antisense oligonucleotides [11]. While miR-21 represents an abundantly expressed miRNA (Figure 1C), miR-18b expression was very low in the cardiomyocytes (Figure 1E). When LNA oligonucleotides antisense (miR-LNA) to miR-21 or miR-18b were transfected into cardiomyocytes, we consistently observed induction of hypertrophy, including increased cardiomyocyte size (Figures 2A and B) and induction of hypertrophic markers such as ANF, α-actinin and skeletal muscle α-actin (Figures 2C-E). Those changes are subtle yet reproducible. The role for miR-18b and 21 in hypertrophy is further supported by a gain-of-function approach whereby we transfected cardiomyocytes with short double-stranded RNAs (miR-duplexes), in which a single-strand identical to mature miR-21 or - 18b is incorporated into the RISC complex [3,12]. Transfection of miR-21 and miR-18b duplexes slightly decreased cardiomyocyte size and decreased expression of hypertrophic markers (Figures 2A-E). Interestingly, transfection of miR-21 duplexes, but not miR-18b duplexes, inhibited PE-induced cardiomyocyte hypertrophy (Figure 2B), suggesting distinct functions for these two miRNAs in hypertrophic growth. Collectively, these data suggest that miR-21 and -18b may play a causative role in cardiomyocyte hypertrophy.

Emerging evidence has pointed to the critical role of miRNAs in variety of biological processes. As an initial step to explore the potential involvement of miRNAs in the pathophysiology of cardiac function, we profiled the changes of miRNA expression in both *in vitro* and *in vivo* cardiac hypertrophy models. Our results demonstrate that many miRNAs are regulated during cardiac hypertrophy and we show that miR-18b and miR-21 play a critical role in the hypertrophic process. Our data are consistent with two recent reports that identified dysregulated miRNA expression during cardiac hypertrophy and documented important roles for miR-1 and miR-195 in cardiac hypertrophy and heart failure [13,14].

We have found that miR-21 expression was induced during hypertrophy and, most importantly, our studies suggest that miR-21 may negatively regulate cardiac hypertrophy. Recent studies have found that miR-21 is likely involved in tumor-related cell growth and apoptosis [4,15, 16]. Interestingly, some of those reports appear contradictory: while one study documented that miR-21 inhibition provoked cell growth in HeLa cells [16], others showed that miR-21 inhibition led to activation of apoptosis and decreased cell proliferation [4,15]. Clearly, the identification of the miR-21 regulatory targets and understanding the molecular pathways modulated by miR-21 in different biological systems will be the key to our understanding the biological function of this miRNA.

ACKNOWLEDGEMENTS

We thank Xiaoyun Hu for excellent technical assistance and Wang lab members for their support. M.T. is a postdoctoral fellow and T. E. C. is a predoctoral fellow of the AHA. This study is supported by the March of Dimes Foundation, Muscular Dystrophy Association and NIH.

Abbreviations

LNA, locked nucleic acid; LIF, leukemia inhibitory factor; miRNA, microRNA; PE, phenylephrine; TAB, thoracic aortic banding.

References

[1]. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell Jan 23;2004 116(2): 281–97. [PubMed: 14744438]

[2]. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, et al. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell Aug 26;2005 122(4):553–63. [PubMed: 16122423]

[3]. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. Cell Oct 17;2003 115(2):199–208. [PubMed: 14567917]

[4]. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, et al. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet Feb;2006 38(2): 228–33. [PubMed: 16380711]

[5]. Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature Jul 14;2005 436(7048):214–20. [PubMed: 15951802]

[6]. Nicol RL, Frey N, Pearson G, Cobb M, Richardson J, Olson EN. Activated MEK5 induces serial assembly of sarcomeres and eccentric cardiac hypertrophy. Embo J Jun 1;2001 20(11):2757–67. [PubMed: 11387209]

[7]. Xing W, Zhang TC, Cao D, Wang Z, Antos CL, Li S, et al. Myocardin induces cardiomyocyte hypertrophy. Circ Res Apr 28;2006 98(8):1089–97. [PubMed: 16556869]

[8]. Thomson JM, Parker J, Perou CM, Hammond SM. A custom microarray platform for analysis of microRNA gene expression. Nat Methods Oct;2004 1(1):47–53. [PubMed: 15782152]

[9]. Thomson JM, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev Aug 15;2006 20(16):2202–7. [PubMed: 16882971]

[10]. Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhelper ME, Field LJ, et al. Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. Proc Natl Acad Sci U S A Sep 15;1991 88(18):8277–81. [PubMed: 1832775]

[11]. Boutz PL, Chawla G, Stoilov P, Black DL. MicroRNAs regulate the expression of the alternative splicing factor nPTB during muscle development. Genes Dev Jan 1;2007 21(1):71–84. [PubMed: 17210790]

[12]. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell Oct 17;2003 115(2):209–16. [PubMed: 14567918]

[13]. Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M. MicroRNAs play an essential role in the development of cardiac hypertrophy. Circ Res Feb 16;2007 100(3):416–24. [PubMed: 17234972]

[14]. van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, et al. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A Nov 28;2006 103(48):18255–60. [PubMed: 17108080]

[15]. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res Jul 15;2005 65(14):6029–33. [PubMed: 16024602]

[16]. Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res 2005;33(4):1290–7. [PubMed: 15741182]

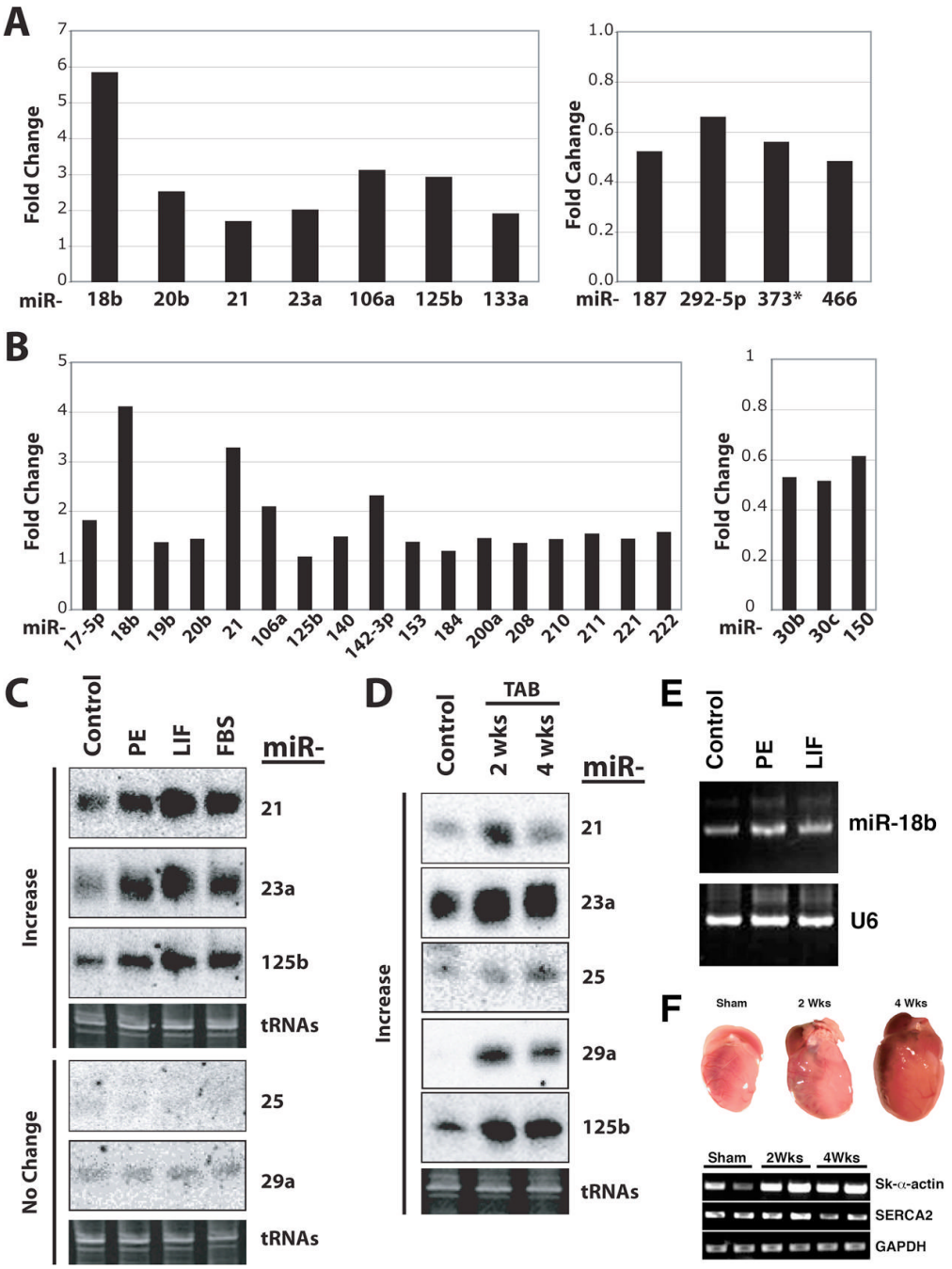

Figure 1.

Microarray screen identifies miRNAs that are regulated during cardiac hypertrophy. (A) Up- and down-regulated miRNAs in phenylephrine (PE) treated neonatal cardiomyocytes. (B) Fold change of miRNA expression in 2 week thoracic aortic banding (TAB) hearts. (C) Northern blot analysis of miRNAs using equal amounts of total RNA isolated from rat neonatal cardiomyocytes treated with various hypertrophic stimuli. PE, phenylephrine; LIF, leukemia inhibitory factor; FBS, fetal bovine serum. (D) Northern blot analysis of miRNAs using equal amounts of total RNA isolated from control, 2 week, or 4 week TAB mice. tRNAs were used as a loading control. (E) RT-PCR analysis for miR-18b expression in PE or LIF treated cardiomyocytes. U6 expression served as control. (F) Gross heart morphology of sham, 2 and

4 week TAB hearts, and RT-PCR analysis for hypertrophic genes using total RNA isolated from those conditions. GAPDH served as control.

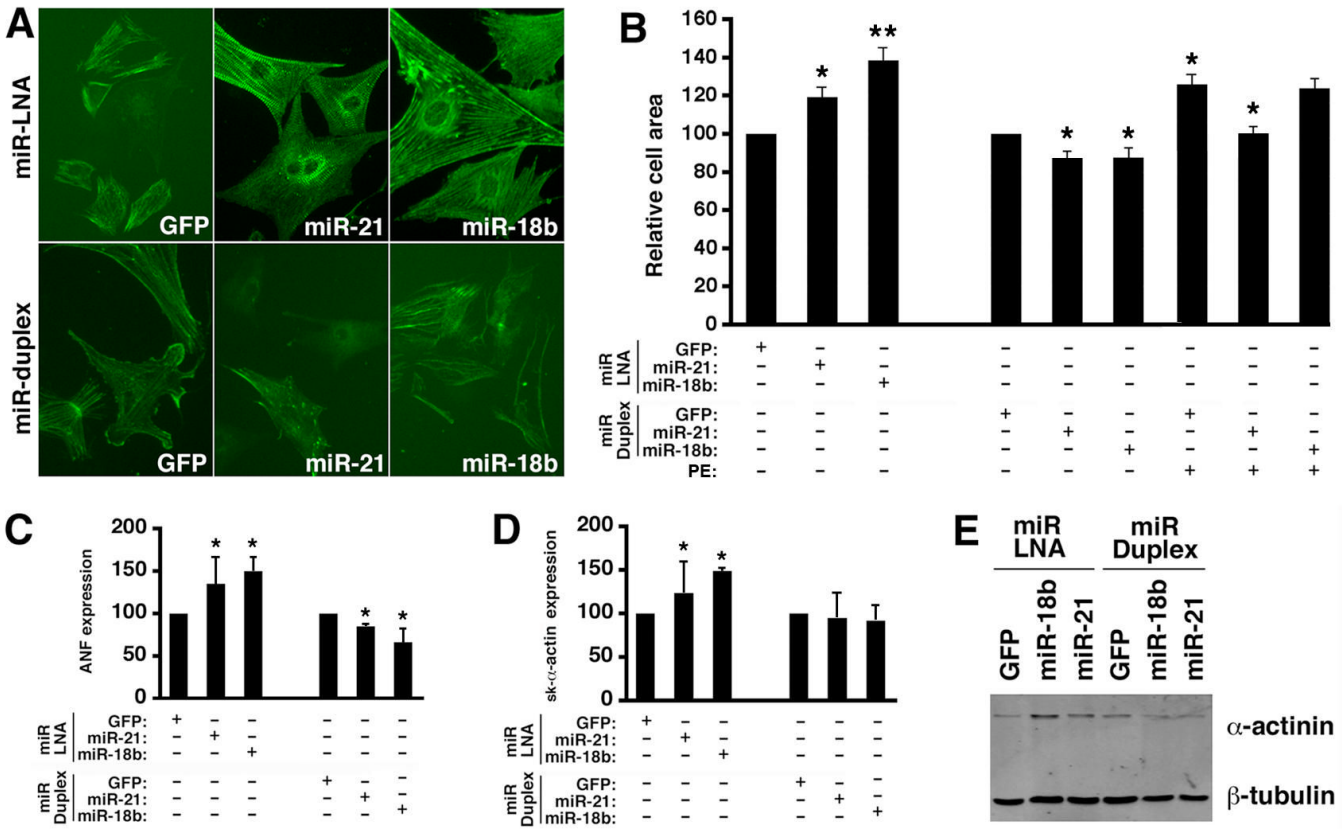

Figure 2.

miR-18b and miR-21 regulate cardiomyocyte hypertrophy. (A) Representative images of α-actinin-stained cardiomyocytes transfected with LNA antisense oligonucleotides against indicated miRNAs (upper panels) or miR-duplexes (lower panels). GFP was used as a control. (B) Quantitative analysis of cardiomyocyte cell size. ~200 cells immunostained with anti-α-actinin antibody from each treatment were randomly chosen for surface area measurement. Results presented as relative cell area compared to controls. (C and D) RT-PCR analysis of hypertrophic marker genes ANF and skeletal muscle α-actin (sk-α-actin) using total RNA isolated from cardiomyocytes treated with indicated LNA oligos, or miRNA duplexes. (E) Western blot analysis of α-actinin expression in cardiomyocytes treated with indicated LNA oligos, miRNA duplexes, or GFP. β-tubulin serves as a loading control. Error bars represent standard deviation; * $p < 0.05$; ** $p < 0.01$.