REGULATION OF EXERCISE-DEPENDENT CARDIAC GROWTH BY MICRORNAS

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DEDICATION

To My Family and Colleagues

For all their support

REGULATION OF EXERCISE-DEPENDENT CARDIAC GROWTH BY MICRORNAS

by

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DISSERTATION

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REGULATION OF EXERCISE-DEPENDENT CARDIAC GROWTH BY MICRORNAS

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The heart is an adaptive organ which undergoes pathological or physiological remodeling in response to a variety of stimuli to meet the demands of the body. Chronic exercise training promotes a physiological remodeling response in which the heart increases in size to match loading demands. In this thesis, I present my studies on the function of microRNAs during exercise-induced cardiac remodeling.

First, I show the expression of muscle-specific microRNA (miRNA), miR-499, is down-regulated by voluntary free-wheel running in hearts of mice. I hypothesized the reduction of miR-499 may be required for exercise-induced cardiac hypertrophy. I found forced cardiac over-expression of miR-499 was associated with diminished physiological cardiac growth, whereas genetic deletion and antimiR mediated inhibition of miR-499 caused enhanced physiological growth following exercise. I also explored the mechanism by which miR-499 represses exercise-induced cardiac growth. I determined the repressive effects of miR-499 are mediated through regulation of IGF-1/PI-3K/Akt and beta-catenin signaling pathways, which drive physiological growth of the heart. I demonstrated the effects of miR-499 on physiological cardiac growth are mediated, at least in part, through repression of a network of genes including p85-alpha, Rictor, Lin7c and Fzd4. Collectively, the results of my thesis research identify miR-499 as a pivotal regulator of exercise-induced cardiac hypertrophy.

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PRIOR PUBLICATIONS

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LIST OF DEFINITIONS

AKT - protein kinase B
ANG II – angiotensin II
AFU - artificial fluorescent units
BW – body weight
cDNA – complementary DNA
CHEK1 – Check point kinase 1
DNA – deoxyribonucleic acid
EGFR1 – epidermal growth factor receptor 1
FZD4 – frizzled 4
GH – Growth Hormone
GPCR – G protein coupled receptor
H&E – hematoxylin and eosin
HRP – horseradish peroxidase
IGF-1 – insulin-like growth factor 1
KB – kilobase
LIN7C – lin7 homolog c
miRNA – microRNA
mTOR – mammalian target of rapamycin
MYH6 – myosin heavy chain 6
MYH7 – myosin heavy chain 7
MYH7B – myosin heavy chain 7B
n.s. – non-signficant
p85α – protein 85 alpha
PBS – phosphate buffered saline
PI3K – phosphatidylinositol 3-kinase
PTU – propylthiouracil
qRT-PCR – quantitative PCR
RAPTOR – regulatory associated protein of mTOR
RICTOR – rapamycin-insensitive companion of mTOR
RISC – RNA-induced silencing complex
RNA – ribonucleic acid
VW – ventricle weight
UTR – untranslated region

Chapter I

Regulation of Stress Induced Cardiac Remodeling

Chapter II

miR-499 Governs Exercise-Dependent Cardiac Growth

ABSTRACT

In response to exercise training, the heart increases in size and function to match physiological demands. Recent reports have implicated microRNAs as key mediators of heart disease, but the potential involvement of microRNAs in physiological cardiac growth has received little attention. Here, we show that microRNA-499 (miR-499), encoded by an intron of a myosin heavy chain gene, is down-regulated in response to exercise training in mice. Forced over-expression of miR-499 in the hearts of transgenic mice impeded physiological cardiac growth in response to exercise, whereas genetic deletion and antimiR inhibition of miR-499 enhanced this process. The suppressive effects of miR-499 on physiological cardiac growth are mediated, at least in part, by repression of multiple components of the IGF-1/Akt and beta-catenin signaling pathways, which drive physiological growth of the heart. These findings reveal a previously unrecognized microRNA signaling circuit whereby miR-499 controls the transmission of intracellular signals that mediate the response of cardiac muscle cells to exercise.

INTRODUCTION

The heart is a highly adaptive organ capable of remodeling in response to a variety of stimuli to meet the demands of the body (Hill and Olson, 2008). Pathological stimuli, including hypertension, myocardial infarction and neurohumoral activation, lead to adverse cardiac remodeling, accompanied by myocyte hypertrophy, apoptosis, and fibrosis, often culminating in systolic dysfunction, electrophysiological abnormalities and heart failure (Chien, 1999; Frey et al., 2004; Haunstetter and Izumo, 2000). In contrast, physiological hypertrophy, as occurs in response to chronic exercise training, pregnancy and normal postnatal growth, preserves cardiac output, without apoptosis or fibrosis (Dorn et al., 2003; Lorell and Carabello, 2000; McMullen and Jennings, 2007).

The downstream signaling cascades associated with pathological and physiological hypertrophy appear to be distinct (Bernardo et al., 2010; Dorn and Force, 2005). Disruption in calcium handling and homeostasis by cardiomyocytes and activation of stress-responsive protein kinase signaling are key features of pathological cardiac hypertrophy (Molkentin et al., 1998; Sadoshima and Izumo, 1997). In contrast, physiological hypertrophy is mediated by insulin-like growth factor-1 (IGF-1) signaling through receptor tyrosine kinase receptors, leading to activation of phosphatidylinositol 3-kinase (PI3K) and subsequent activation of Akt signaling (Matsui et al., 2003; Shiojima and Walsh, 2006).

MicroRNAs (miRNAs) are small, non-protein coding RNA molecules that decrease mRNA stability and/or translation through complimentary base pairing to the 3' untranslated regions (UTRs) of target mRNAs (Ambros, 2004; Bartel, 2004). miRNAs have been implicated in cardiomyocyte survival, angiogenesis, fibrosis, growth and contractility during pathological cardiac remodeling (Bauersachs and Thum; Latronico and Condorelli; Small and Olson, 2011). However, the potential role of miRNAs in physiological cardiac growth has not been fully explored.

Previously, a network of myosin encoded microRNAs, termed the myomiRs were identified as key mediators of cardiac remodeling in response to pressure overload (van Rooij et al., 2009). The myomiRs consists of a group of three microRNAs including miR-208a, miR-208b and miR-499 located within the introns of Myh6, Myh7 and Myh7B, respectively. This study demonstrated the expression of miR-208a is required for the heart to revert to a fetal gene program in response to pathological stimuli and its effects are mediated through indirect regulation of Myh7/miR-208b and Myh7B/miR-499 (van Rooij et al., 2009). Interestingly, re-expression of miR-499 alone was sufficient to allow normal re-activation of fetal genes, suggesting miR-499 is required for pathological remodeling of the heart.

In the present study, we show that miR-499 is down-regulated in the heart in response to exercise training in mice. Transgenic over-expression of miR-499

blocks exercise-induced cardiac growth while genetic and antimiR-mediated knockdown of miR-499 augments the cardiac growth response following exercise. MiR-499 appears to control physiological cardiac hypertrophy, at least in part, through repression of multiple components of the PI3K-Akt and beta-catenin signaling pathways (Xu et al., 2004), which are required for exercise-induced physiological growth of the heart. These findings demonstrate a novel role for miR-499 as a modulator of cardiac muscle response to exercise by regulating multiple hypertrophy signaling cascades.

RESULTS

miRNA regulation in response to exercise

We examined whether miRNAs are regulated in the heart in response to exercise training by subjecting adult male mice to a voluntary free wheel running regime for 10 days ($n \geq 5$ for each group). At the end of the training period, ventricular weight versus total body weight (VW/BW) was compared in sedentary and exercised animals as a measure of physiological hypertrophy. Exercised mice showed a statistically significant increase in VW/BW compared with sedentary mice (Figure 2.2 A; $p<0.05$). The left ventricles from sedentary and exercised mice were analyzed by miRNA microarray, which revealed 26 miRNAs that were up-regulated and 41 that were down-regulated (Table 2.1). To narrow our focus, we examined miRNAs that showed a greater than two-fold change and had a

signal intensity of 1000 artificial fluorescent units (AFU) or greater. Applying these cut-offs, we identified a group of 12 regulated miRNAs, (Figure 2.2 B). Among these, we removed miR-126-5p and miR-145 from further analysis because they are expressed specifically in endothelial and vascular smooth muscle cells, respectively (Wang et al., 2008; Xin et al., 2009). We confirmed the changes in expression of multiple miRNAs by qRT-PCR (Figure 2.1).

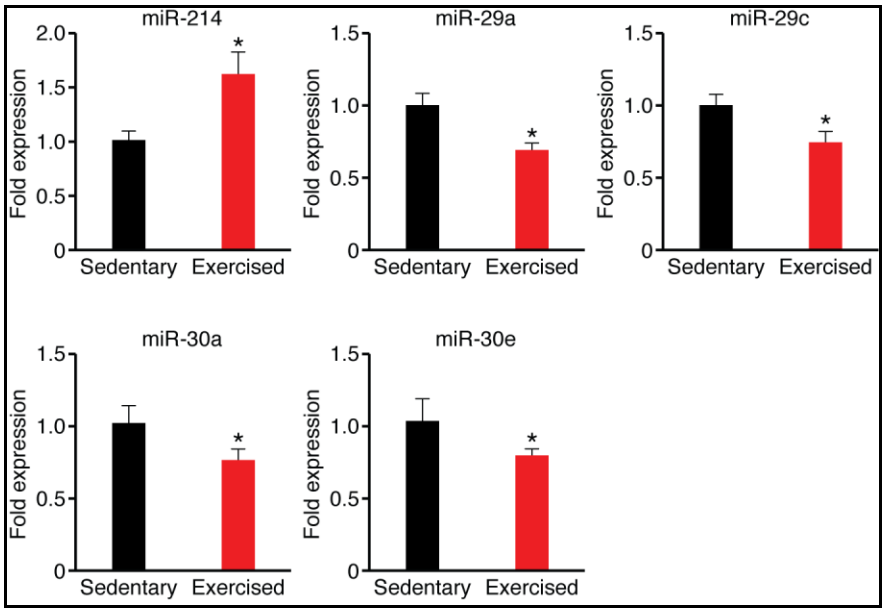

Figure 2.1: Confirmation of microRNAs microarray. qRT-PCR analysis of miRNAs identified by microarray analysis of ventricles for sedentary mice and mice after 10 days of voluntary exercise training.

Probe_ID	Sedentary	Exercise	Fold Change
mmu-miR-690	459.83	4,036.19	8.77757
mmu-miR-132	284.14	1,000.03	3.519497
mmu-miR-709	17,107.87	57,193.36	3.343102
mmu-miR-214	1,050.97	3,345.64	3.183383
mmu-miR-200c	43.87	139.51	3.180078
mmu-miR-574-5p	81.1	215.17	2.653144
mmu-miR-497	144.35	357.88	2.479252
mmu-miR-145	5,881.77	12,752.45	2.168131
mmu-miR-1224	1,516.09	2,868.48	1.892025
mmu-miR-762	3,372.19	6,355.86	1.884787
mmu-miR-320	1,084.98	1,983.33	1.827988
mmu-miR-705	4,218.99	7,677.83	1.819827
mmu-miR-99b	415.66	723.11	1.739667
mmu-miR-107	570.01	941.95	1.652515
mmu-miR-423-5p	398.63	631.17	1.583348
mmu-miR-103	601.83	952.27	1.582291
mmu-miR-361	920.45	1,408.48	1.530208
mmu-miR-21	5,467.06	8,076.11	1.477231
mmu-miR-92a	1,603.22	2,327.02	1.451466
mmu-miR-20a	421.75	601.28	1.425679
mmu-miR-805	9,606.85	13,554.61	1.410932
mmu-let-7b	20,441.75	27,469.35	1.343787
mmu-let-7e	16,591.49	21,288.97	1.283126
mmu-let-7a	27,983.44	35,607.75	1.272458
mmu-miR-486	6,447.44	8,202.60	1.272226
mmu-miR-151-5p	2,340.91	2,883.09	1.231611
mmu-miR-133a	13,646.31	11,311.32	0.828892
mmu-miR-1	45,502.98	37,415.48	0.822264
mmu-let-7i	10,990.84	8,955.55	0.814819
mmu-miR-133b	13,779.73	10,723.05	0.778176
mmu-miR-451	4,069.13	3,087.04	0.758649
mmu-miR-100	1,317.33	989.2	0.750913
mmu-miR-16	10,215.95	7,631.37	0.747005
mmu-let-7g	13,289.46	9,473.53	0.71286
mmu-miR-126-3p	21,611.10	14,911.66	0.69
mmu-miR-26a	19,141.97	12,622.43	0.659411

mmu-miR-195	6,007.58	3,807.54	0.633789
mmu-miR-30d	3,967.40	2,408.65	0.60711
mmu-miR-152	989.6	574.2	0.580234
mmu-miR-223	366.71	210.93	0.575196
mmu-miR-26b	13,044.26	7,502.63	0.575167
mmu-miR-27a	5,124.84	2,734.01	0.533482
mmu-miR-139-5p	771.37	403.37	0.522927
mmu-miR-143	3,476.33	1,759.44	0.50612
mmu-miR-27b	6,501.07	3,263.77	0.502036
mmu-miR-30a*	1,036.31	514.39	0.496367
mmu-miR-29a	9,467.34	4,655.76	0.491771
mmu-miR-30a	4,906.28	2,309.57	0.470738
mmu-miR-30e	2,931.73	1,374.08	0.468693
mmu-miR-10a	151.63	66.47	0.43837
mmu-miR-322	968.12	387.23	0.399981
mmu-miR-10b	228.49	88.4	0.386888
mmu-miR-499	3,919.34	1,419.27	0.36212
mmu-miR-15a	1,781.92	625.18	0.350846
mmu-miR-374	145.93	48.33	0.331186
mmu-miR-30e*	1,029.74	340.73	0.330889
mmu-miR-181d	118.52	39.21	0.33083
mmu-miR-29c	4,923.07	1,552.57	0.315366
mmu-miR-101b	126.02	37.67	0.298921
mmu-miR-148a	437.01	120.1	0.274822
mmu-miR-181b	125.35	31.82	0.253849
mmu-miR-145*	146.3	32.72	0.22365
mmu-miR-150	3,176.86	2,760.52	0.21
mmu-miR-101a	256.03	50.11	0.195719
mmu-miR-126-5p	3,567.13	677.05	0.189802
mmu-miR-29b	615.67	85.28	0.138516
mmu-miR-146b	119.94	15.67	0.130649

Table 2.1 miRNAs regulated in response to exercise training.
List of microRNAs significantly regulated in hearts of mice after 10 days of voluntary exercise compared to sedentary mice.
Call list (differentially expressed transcripts with p-value < 0.01)

Of the miRNAs that were regulated in response to exercise training, miR-499 was previously identified as an important regulator of pathological cardiac remodeling (Shieh et al., 2011; van Rooij et al., 2009; Wang et al., 2011). To confirm the down-regulation of miR-499 in response to exercise, we analyzed expression of miR-499 and its host gene, Myh7b, after 3, 14 and 28 days of exercise in additional cohorts of mice (n=5 for each group). After 3 days of exercise, levels of miR-499 and Myh7b were not significantly changed compared to sedentary mice. However, after 14 and 28 days of exercise training, both miR-499 and Myh7b were down-regulated ~2-fold compared to sedentary age-matched controls (Figure 2.2 C). These findings suggested that miR-499, which is expressed specifically in cardiomyocytes and not in other cells of the heart (e.g. fibroblasts and endothelial cells), might play a role in physiological cardiac growth.

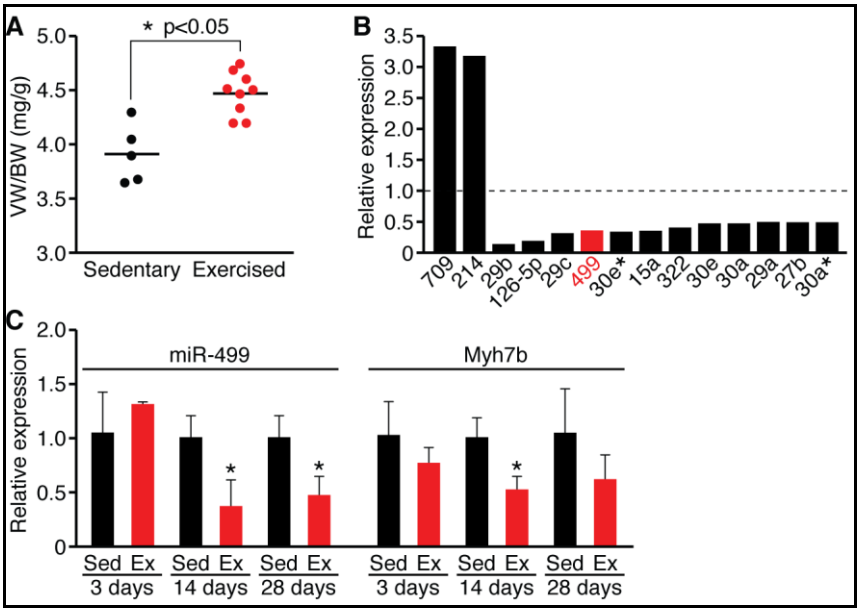

Figure 2.2: Changes in expression of specific cardiac miRNAs in response to voluntary exercise training. (A) Ratio of ventricular weight to body weight (VW/BW) in sedentary mice and mice subjected to 10 days of voluntary wheel running exercise (n≥5 per group). Each data point represents an individual mouse. (B) miRNA microarray expression profile of heart tissue between sedentary mice and after exercise training. (C) qRT-PCR analysis of miR-499 and host gene *Myh7b* expression under sedentary (Sed) conditions and after 3, 14 and 28 days of voluntary exercise (Ex). * $p<0.05$

Cardiac over-expression of miR-499 impairs cardiac growth in response to exercise.

To investigate the potential involvement of miR-499 in physiological cardiac growth in response to exercise, we exercised miR-499 transgenic (TG) mice, in which cardiac expression of miR-499 was increased ~3-fold under control of the muscle creatine kinase (MCK) promoter (van Rooij et al., 2009). Under baseline conditions at 8 weeks of age, miR-499TG mice showed no statistically significant difference in VW/BW compared to wildtype (WT)

littermates (Figure 2.3 A). However, in response to 14 days of exercise training, TG mice failed to display an increase in heart size (Figure 2.3 A), despite exercising at comparable levels to WT controls (Figure 2.3 B). Analysis of cardiac output before and after exercise training showed preserved function between WT and TG mice (Figure 2.3 C). Measurements of the cross-sectional areas of cardiomyocytes in histological sections of hearts of WT and TG hearts also showed a block to myocyte hypertrophy in TG mice in response to exercise (Figure 2.3 D). We found no evidence of cell death or proliferation in response to exercise training in either cohort (Figure 2.4 A-B). These findings suggested that miR-499 blunts myocyte hypertrophy in response to exercise, suggesting that down-regulation of miR-499 is an adaptive mechanism to promote physiological cardiac growth.

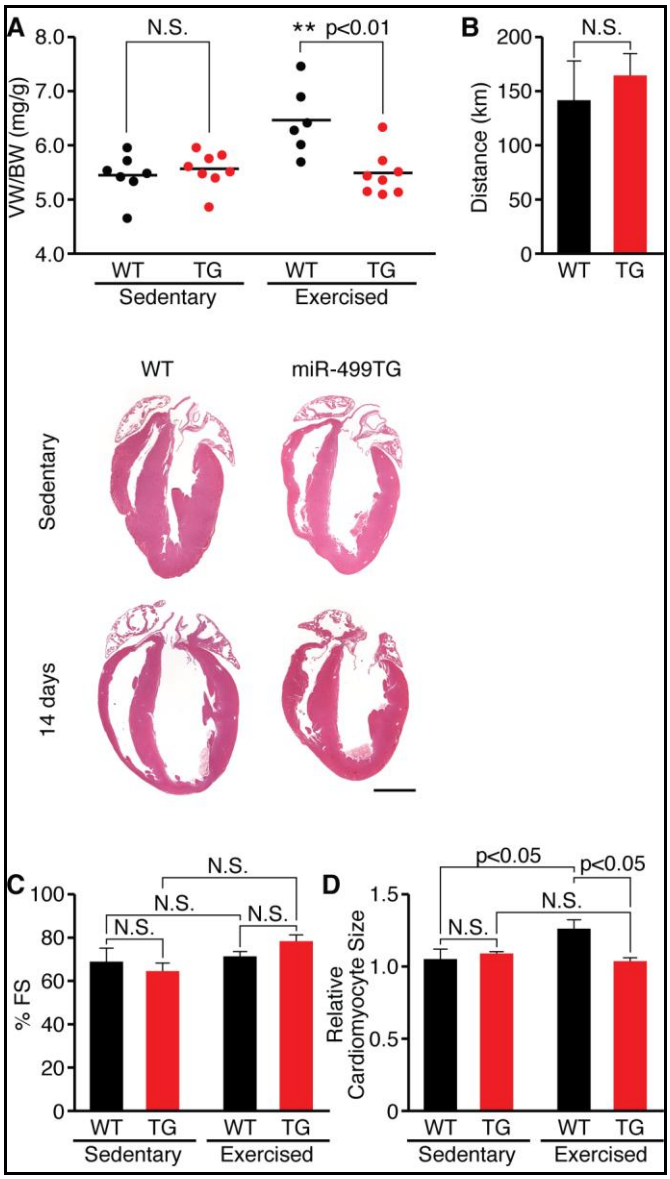

Figure 2.3: Overexpression of miR-499 blocks exercise induced cardiac hypertrophy. (A) Ratio of ventricular weight to body weight (VW/BW) under sedentary conditions and following exercise for 14 days in WT and TG mice. Each data point represents an individual mouse. H&E stained heart sections from WT and TG mice under sedentary conditions and after two weeks of exercise training. Scale bar = 2mm. (B) Average total distance run by WT and TG mice during 2 weeks of exercise training (n≥6 per group). (C) Percent fractional shortening (FS) before and after exercise of WT and TG mice. (D) Relative size

of cardiomyocytes in sedentary and exercised mice. Relative cardiomyocyte size was calculated by examining cross-sectional area of individual myocytes using wheat germ agglutinin staining. (n=3 per group, minimum 500 cells analyzed per mouse)

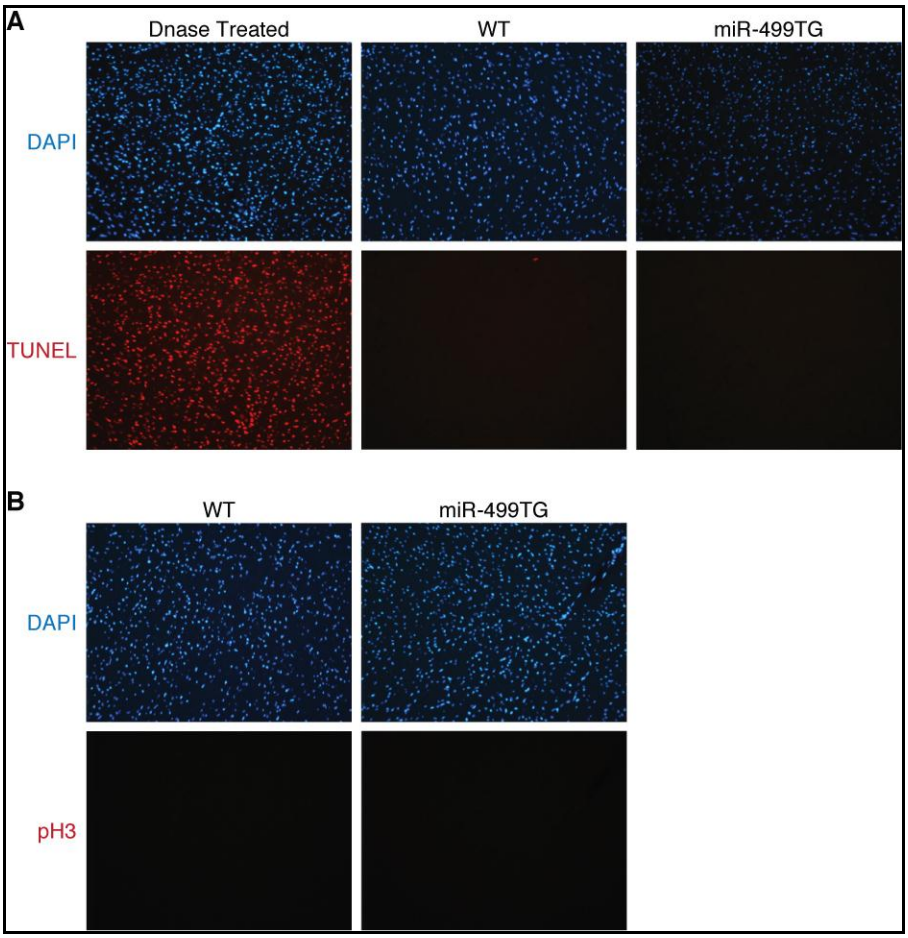

Figure 2.4: Analysis of proliferation and apoptosis after exercise training in miR-499TG mice. (A) Immuno-histochemical analysis for apoptosis in WT and TG heart sections in response to exercise. DAPI staining visualizes the nucleus and TUNEL positive cells indicated DNA cleavage. Dnase was applied as a positive control. (B) Analysis of proliferation was examined using phospho-histone H3 staining.

Genetic deletion of miR-499 enhances cardiac growth in response to exercise.

We next investigated the effect of genetic deletion of miR-499 on the response of the heart to exercise training. At baseline, miR-499 knockout (KO) mice were phenotypically normal and showed no differences in heart size or function compared to WT mice (Figure 2.5 A). In response to 14 days of exercise training, both WT and KO mice underwent cardiac hypertrophy (n=4 for each group). However, KO mice showed a greater increase in VW/BW in response to exercise training compared to WT mice (49% vs 28% increase; $p \leq 0.05$) (Figure 2.5 A). There was no difference in total distance run by the two groups, indicating that KO mice are capable of exercising at levels similar to WT mice (Figure 2.5 B). Gross morphological analysis of the hearts after exercise training also showed no evidence of pathological remodeling (Figure 2.5 A) and echocardiography revealed preserved cardiac function between WT and KO mice under either sedentary or exercise conditions (Figure 2.5 C).

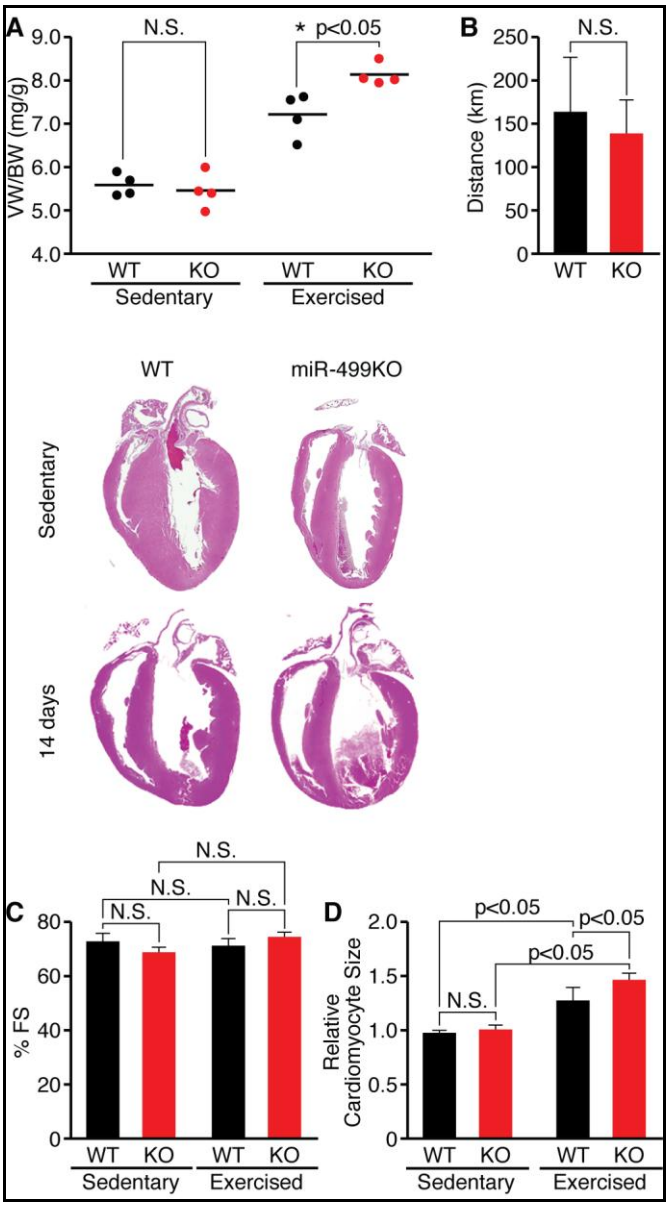

Figure 2.5: miR-499 knockout mice exhibit increased cardiac hypertrophy in response to exercise. (A) Ratio of ventricular weight to body weight (VW/BW) under sedentary conditions and following exercise conditions in WT and KO mice. Each data point represents an individual mouse. H&E stained heart sections from WT and KO mice under sedentary conditions and after two weeks of exercise training. Scale bar = 2mm. (B) Average total distance run by WT and KO mice during 14 days of exercise training. (n=4 per group) (C) Fractional shortening (FS) before and after exercise of WT and KO mice. (D) Relative

cardiomyocyte size in sedentary and exercised mice. Relative cardiomyocyte was calculated by examining cross-sectional area of individual myocytes using wheat germ agglutinin staining. (n=3 per group, minimum 500 cells analyzed per mouse)

Examination of WT and KO mice after exercise showed no differences in cardiomyocyte proliferation or apoptosis, which could account for the differences observed in heart size (Figure 2.6 A-B). However, examination of cardiomyocyte size showed that KO mice had larger cardiomyocytes compared to WT mice after exercise (Figure 2.5 D). These results imply that the genetic loss of miR-499 leads to enhanced cardiomyocyte hypertrophy in response to exercise training.

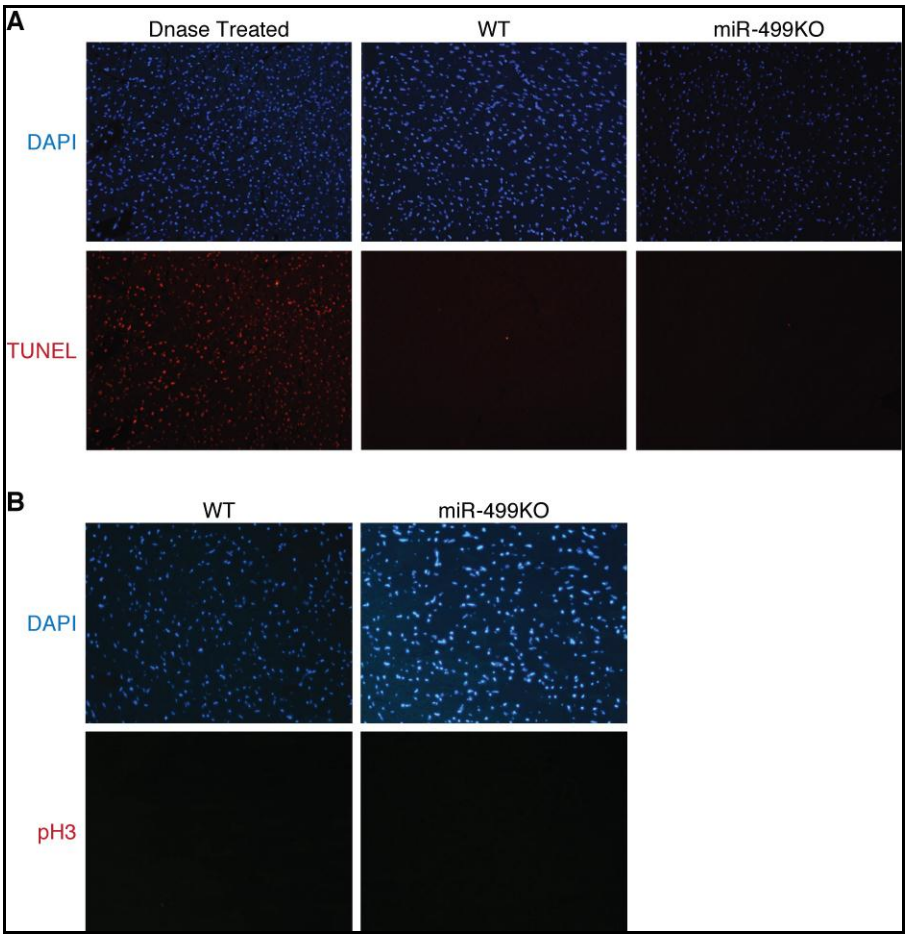

Figure 2.6: **Analysis of proliferation and apoptosis after exercise training in miR-499KO mice.** (A) Immuno-histochemical analysis for apoptosis in WT and KO heart sections in response to exercise. DAPI staining visualizes the nucleus and TUNEL positive cells indicated DNA cleavage. Dnase was applied as a positive control. (B) Analysis of proliferation was examined using phospho-histone H3 staining.

AntimiR knockdown of miR-499 enhances cardiac growth in response to exercise.

We next investigated whether acute repression of miR-499 in the adult heart using an antimiR could also enhance exercise-dependent cardiac growth. To

address this question, we used a 16mer locked nucleic acid (LNA) antimiR specific to miR-499 (antimiR-499), as well as a universal 16mer LNA-modified oligonucleotide as a control (antimiR-control), which targets a miRNA present in *C. elegans* but not found in mammals (Porrello et al., 2011b). AntimiRs were delivered to adult male mice by tail vein injection at a dose of 10mg/kg for 3 days and assayed for efficiency of miR-499 knock-down 48 hours after the last antimiR dose (Figure 2.7 A). By northern blot, miR-499 was undetectable in the hearts of antimiR-499 treated animals (Figure 2.7 B). By qRT-PCR analysis, the knockdown of miR-499 was greater than 90% in the antimiR-499 group after 48 hours and the effect was persistent 14 days post-injection compared to the control group (Figure 2.7 C). This effect was only seen in the heart and expression of miR-499 was not altered in slow skeletal muscle, in which antimiR uptake and inhibition is relatively inefficient.

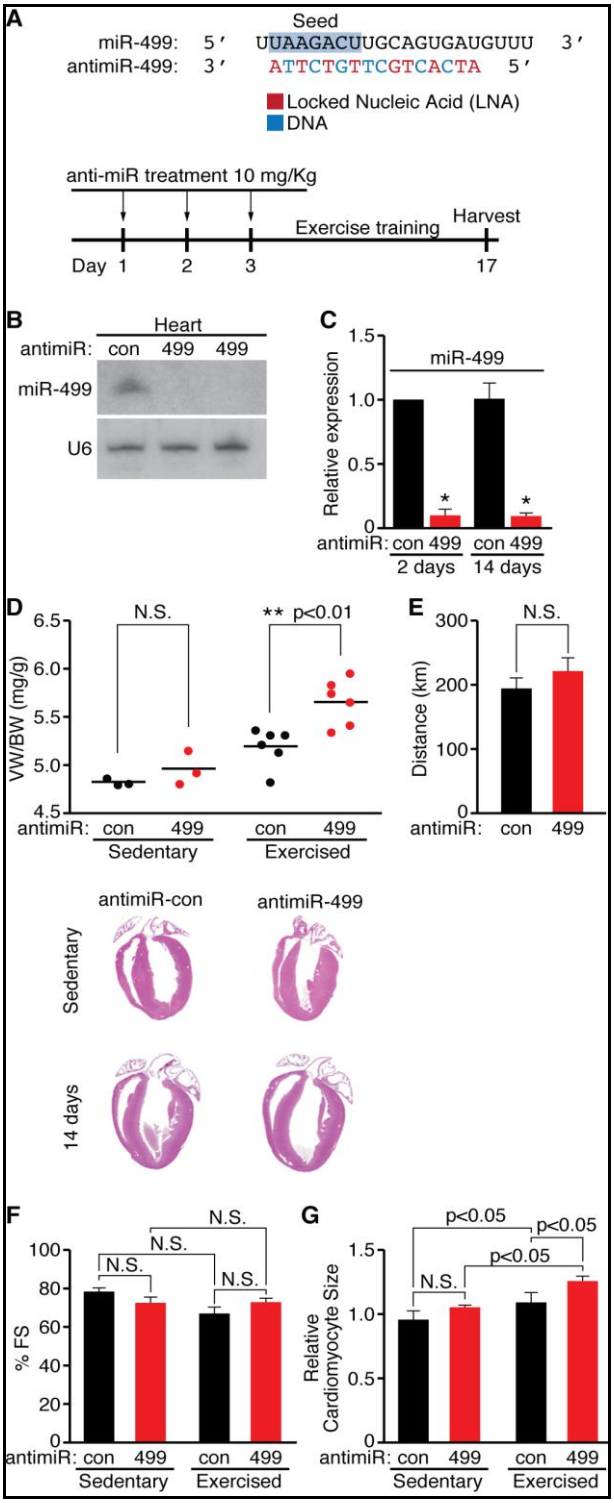
A
Seed
miR-499: 5′ UUAAGACUUGCAGUGAUGUUU 3′
antimiR-499: 3′ ATTCTGTTCGTCACTA 5′
Locked Nucleic Acid (LNA)
DNA
anti-miR treatment 10 mg/Kg
Exercise training
Harvest
Day 1 2 3 17
B
Heart
antimiR: con 499 499
miR-499
U6
C
miR-499
Relative expression
1.5
1.0
0.5
0
antimiR: con 499 con 499
2 days 14 days
D
N.S.
** p<0.01
VW/BW (mg/g)
6.5
6.0
5.5
5.0
4.5
antimiR: con 499 con 499
Sedentary Exercised
E
N.S.
Distance (km)
300
200
100
0
antimiR: con 499
antimiR-con
antimiR-499
Sedentary
14 days
F
N.S.
N.S.
N.S.
N.S.
% FS
100
80
60
40
20
0
antimiR: con 499 con 499
Sedentary Exercised
G
p<0.05
p<0.05
p<0.05
N.S.
Relative Cardiomyocyte Size
1.5
1.0
0.5
0
antimiR: con 499 con 499
Sedentary Exercised

Figure 2.7: AntimiR-mediated knockdown of miR-499 enhances cardiac hypertrophy after exercise. (A) Sequence of miR-499 and LNA-modified antimiR. LNA modified nucleotides are indicated in red. Time course outlining administration of antimiR and exercise training. (B) Northern blot analysis for knockdown of miR-499 2 days after last administration of antimiR. U6 was used as a loading control. (C) Real Time RT-PCR for miR-499 expression after antimiR-499 treatment. (n=4 per group) (D) Ratio of ventricular weight to body weight (VW/BW) under sedentary conditions and after 14 days of exercise training in antimiR control and antimiR-499 treated mice. ($n \geq 3$ per group) Each data point represents an individual mouse. H&E stained heart sections under sedentary conditions and after exercise. (E) Average total distance run by antimiR-con and antimiR-499 treated mice during 14 days of exercise training. (F) Fractional shortening (FS) after antimiR treatment under sedentary and exercised conditions. (G) Relative cardiomyocyte size in sedentary and exercised mice. Relative cardiomyocyte was calculated by examining cross-sectional area of individual myocytes using wheat germ agglutinin staining. (n=3 per group, minimum 500 cells analyzed per mouse)

Under sedentary conditions, we found no significant differences in VW/BW between the two groups 17 days after injection (Figure 2.7 D). However, in response to exercise training, antimiR-499 treated mice displayed an increase in VW/BW compared anti-control treated mice (Figure 2.7 D; $p<0.01$). Examination of antimiR-control and antimiR-499 treated animals showed no evidence of pathological remodeling under sedentary or exercise conditions (Figure 2.7 D). There was no significant difference in the average total distance run by the two groups (Figure 2.7 E). Analysis of percent fractional shortening between antimiR-control and antimiR-499 cohorts under sedentary and exercised conditions also revealed preserved cardiac function between any of the groups (Figure 2.7 F). We did not identify any significant differences in cardiomyocyte apoptosis or

proliferation between the two groups (Figure 2.8 A-B). In response to exercise training, cardiomyocytes in the antimiR-499 cohort showed increased size relative to the antimiR-control treated group (Figure 2.7 G). Thus, acute knockdown of miR-499 is capable of allowing enhanced cardiomyocyte growth in response to exercise training.

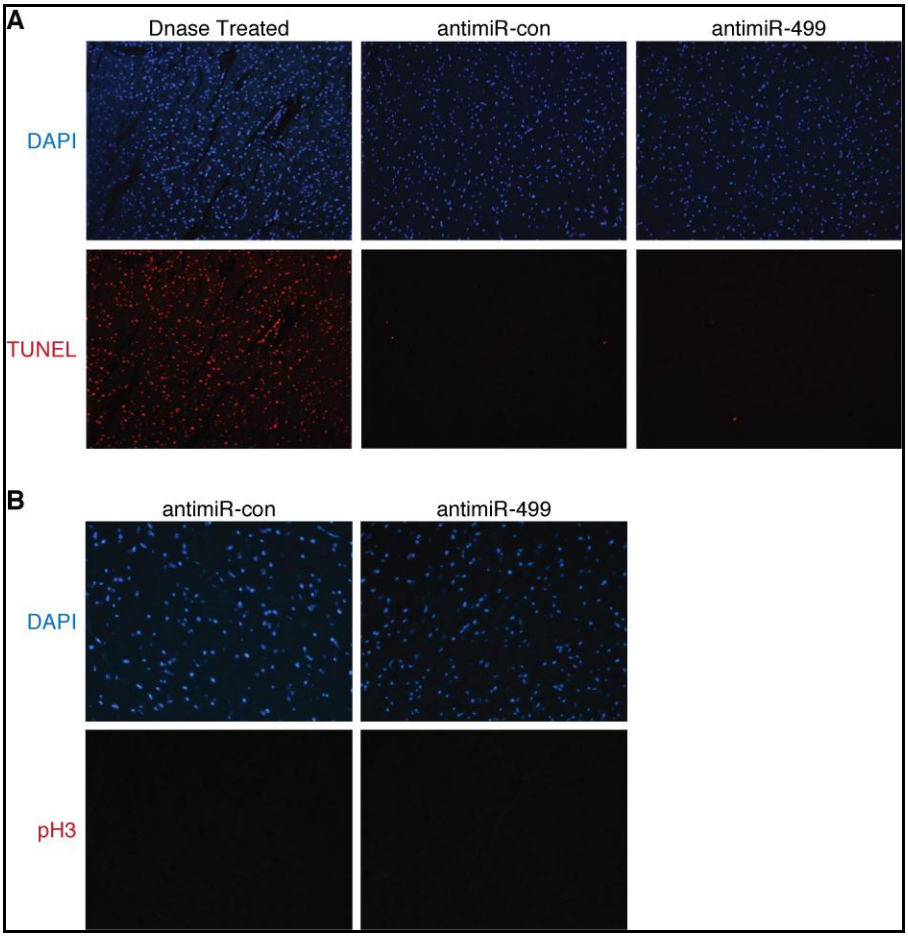

Figure 2.8: Analysis of proliferation and apoptosis after exercise training in antimiR-499 treated mice. (A) Immuno-histochemical analysis for apoptosis in antimiR-control and antimiR-499 treated heart sections in response to exercise.

DAPI staining visualizes the nucleus and TUNEL positive cells indicated DNA cleavage. Dnase was applied as a positive control. (B) Analysis of proliferation was examined using phospho-histone H3 staining.

Modulation of miR-499 influences phosphorylation of Akt in response to exercise.

Akt signaling promotes exercise-induced cardiac hypertrophy (McMullen and Jennings, 2007). IGF-1 signaling leads to activation of the PI3K pathway with subsequent phosphorylation of Akt on serine 473, thereby activating a signaling cascade leading to myocyte hypertrophy (DeBosch et al., 2006). To begin to decipher the mechanistic basis of the anti-hypertrophic actions of miR-499, we tested whether modulation of miR-499 affects the ratio of phosphorylated Akt to total Akt in response to exercise training. After 14 days of exercise, total Akt levels were not different between WT and TG mice. However, over-expression of miR-499 suppressed cardiac Akt phosphorylation (Figure 2.9 A). Conversely, KO and antimiR-499 treated mice showed an increase in p-Akt without a change in total Akt protein (Figure 2.9 B-C). The ratio of p-Akt/total Akt was reduced by half in the TG mice after exercise and was increased in both KO and antimiR-499 treated animals (Figure 2.9 D). These results suggest that miR-499 is capable of suppressing Akt signaling in response to exercise.

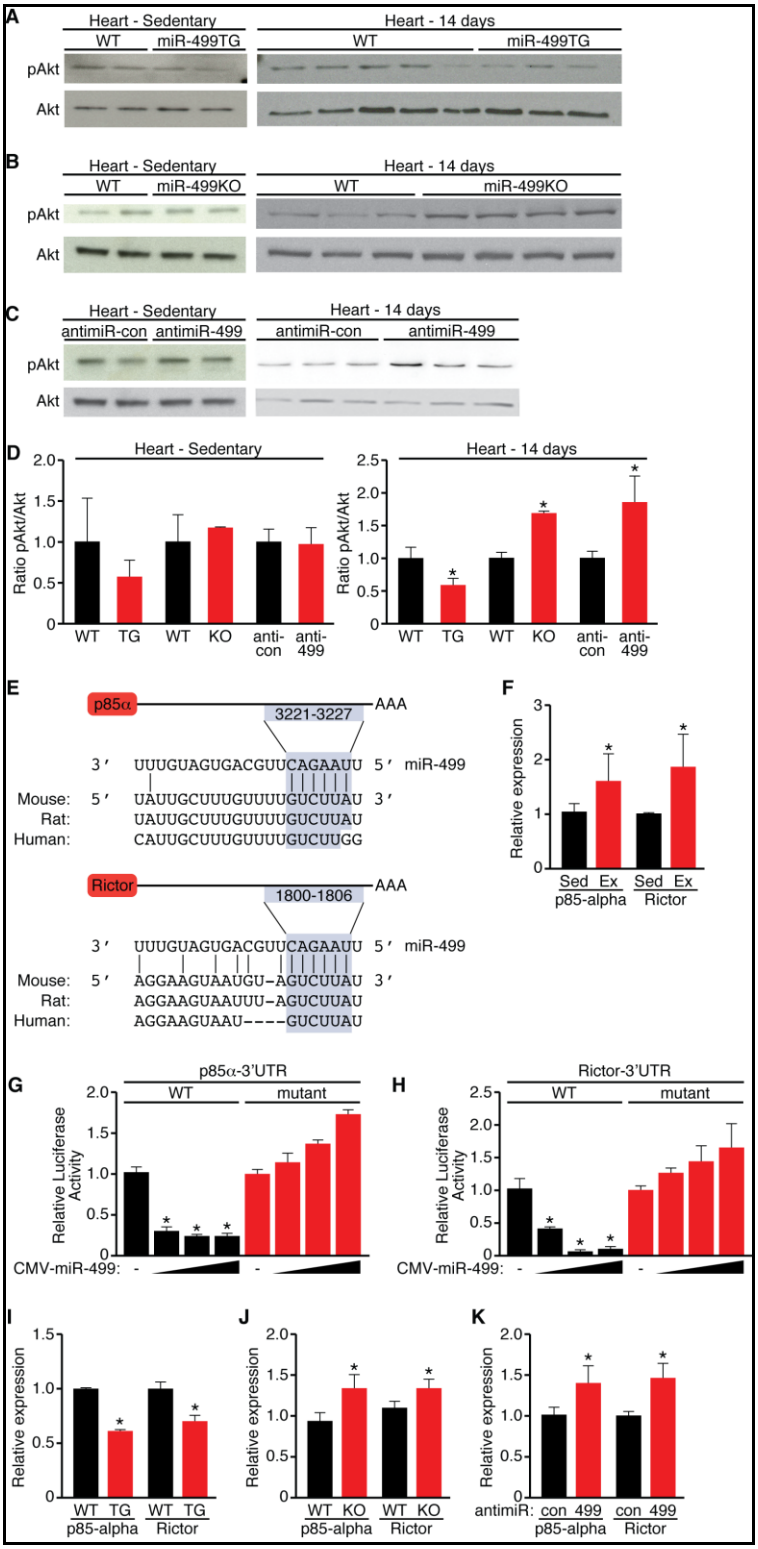
A
Heart - Sedentary
WT
miR-499TG
Heart - 14 days
WT
miR-499TG
pAkt
Akt
B
Heart - Sedentary
WT
miR-499KO
Heart - 14 days
WT
miR-499KO
pAkt
Akt
C
Heart - Sedentary
antimiR-con
antimiR-499
Heart - 14 days
antimiR-con
antimiR-499
pAkt
Akt
D
Heart - Sedentary
Heart - 14 days
Ratio pAkt/Akt
WT TG WT KO anti-con anti-499
E
p85α
AAA
3221-3227
3′ UUUGUAGUGACGUUCAGAAUU 5′ miR-499
Mouse: 5′ UAUUGCUUUGUUUUGUCUUAU 3′
Rat: UAUUGCUUUGUUUUGUCUUAU
Human: CAUUGCUUUGUUUUGUCUUGG
Rictor
AAA
1800-1806
3′ UUUGUAGUGACGUUCAGAAUU 5′ miR-499
Mouse: 5′ AGGAAGUAAUGU-AGUCUUAU 3′
Rat: AGGAAGUAAUUU-AGUCUUAU
Human: AGGAAGUAAU----GUCUUAU
F
Relative expression
Sed Ex Sed Ex
p85-alpha Rictor
G
p85α-3'UTR
WT
mutant
Relative Luciferase Activity
CMV-miR-499: - -
H
Rictor-3'UTR
WT
mutant
Relative Luciferase Activity
CMV-miR-499: - -
I
Relative expression
WT TG WT TG
p85-alpha Rictor
J
Relative expression
WT KO WT KO
p85-alpha Rictor
K
Relative expression
antimiR: con 499 con 499
p85-alpha Rictor

Figure 2.9: miR-499 Suppresses Akt phosphorylation through direct repression of known Akt mediators. (A-C) Western blots of cardiac ventricle tissue are shown under sedentary conditions and after 14 days of exercise training. (A) Total Akt and Phosphorylation of Akt at Ser-473 (pAkt) in TG mice compared to WT controls. (B) Total Akt and pAkt in KO compared to WT controls after exercise (C) Total Akt and pAkt in antimiR-499 treated mice compared to control treated mice (antimiR-con). (D) Quantification of the ratio of pAkt total Akt levels. (E) Location and conservation of predicted miR-499 binding sites in the 3'UTR of PI3K p85 alpha (p85α) and rapamycin-insensitive companion of mTOR (Rictor). Homology to the seed sequence is indicated in blue. (F) Real Time RT-PCR analysis of transcripts encoding p85α and Rictor in hearts of mice under sedentary conditions and after 14 days of exercise. (G-H) Luciferase assay with the pmiR construct driving luciferase expression with a CMV promoter linked to the 3'UTR of each target gene shows repression with increasing expression of miR-499. Mutation of the sites blunts repression. (I-K) Expression of predicted miR-499 target genes after 14 days of exercise in hearts of WT and TG mice, WT and KO mice as well as antimiR-control versus antimiR-499 treated animals. * $p<0.05$.

To identify the possible targets of miR-499 that mediate its regulation of Akt signaling and cardiac hypertrophy, we examined bioinformatics prediction programs for predicted miR-499 targets with potential roles in Akt signaling. We identified conserved miR-499 binding sites in mRNAs encoding two known regulators of Akt signaling: PI3K p85 alpha (p85α) and rapamycin-insensitive companion of mTOR (Rictor) (Figure 2.9 E). Transcript p85α and Rictor were increased by 14 days of exercise (Figure 2.9 F), consistent with a repressive influence of miR-499 on their expression. Moreover, miR-499 repressed expression of a luciferase reporter linked to the p85α and Rictor 3' UTRs in a dose-dependent manner. Mutation of nucleotides 2 and 3 of the miRNA binding site ablated the repression of both targets by miR-499 (Figure 2.9 G-H). After 14

days of exercise training, TG mice had reduced expression of p85α and Rictor (Figure 2.9 I), whereas genetic deletion and knockdown of miR-499 led to an increase in expression of both genes (Figure 2.9 J-K). These results identify two direct targets of miR-499 capable of regulating PI3K and Akt signaling in response to exercise.

Modulation of miR-499 influences expression of genes involved in beta-catenin signaling.

In addition to the well-established role of Akt signaling in the control of muscle growth (McMullen and Jennings, 2007), recent studies have implicated beta-catenin signaling in myocyte hypertrophy (Haq et al., 2003; Li et al.). Previous *in silico* analysis of predicted miR-499 target genes identified an enrichment of Wnt/beta-catenin genes (Wilson et al., 2010). Among the strongly predicted targets of miR-499 are two regulators of the Wnt/beta-catenin pathway, frizzled-4 (Fzd4) and lin7 homolog c (Lin7c) (Onda et al., 2007; Zhang et al.). In response to 14 days of exercise training, Fzd4 and Lin7c transcripts were up-regulated compared to sedentary controls (Figure 2.10 A). Moreover, these potential miR-499 targets were down-regulated in TG mice after exercise (Figure 2.10 B). In contrast, KO and antimiR-499 treated mice subjected to exercise training showed increased expression of Fzd4 and Lin7c (Figure 2.10 C-D).

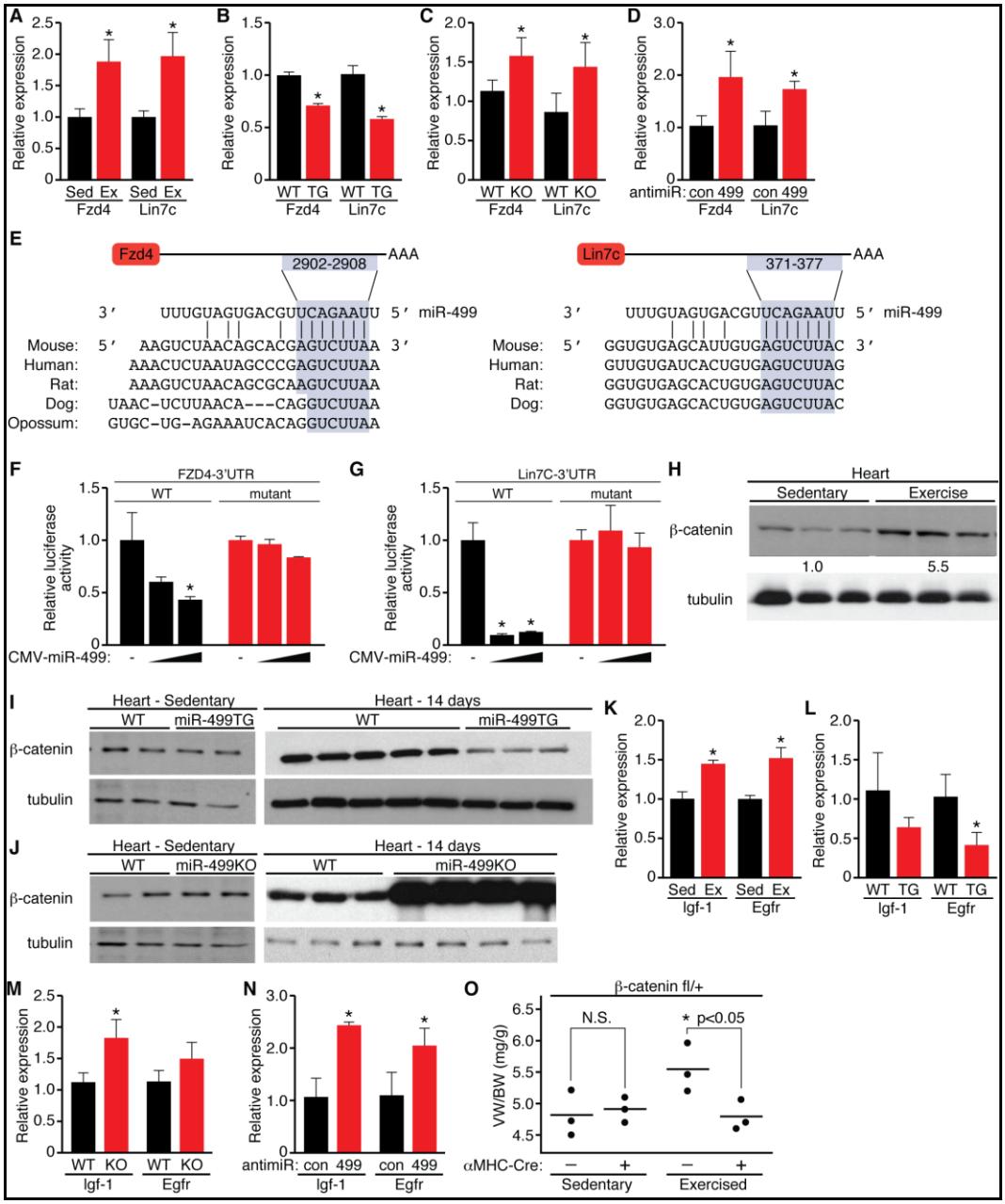

Figure 2.10: Regulation of miR-499 target genes and Beta-Catenin by exercise. We identified genes with predicted miR-499 binding sites that had been reported to be up-regulated in heart by exercise training (A) Real Time RT-PCR analysis of transcripts encoding frizzled 4 (Fzd4) and lin homolog 7c (Lin7c) in hearts of mice under sedentary conditions and after 14 days of exercise. (B-D) Expression of predicted miR-499 target genes after 14 days of exercise in hearts

of WT and TG mice, WT and KO mice, as well as antimiR-control versus antimiR-499 treated animals. (E) Conserved base pairings for miR-499 present in the 3'UTR of the predicted target genes. Homology to seed sequence is indicated in blue. (F-G) Luciferase assay with the pmiR construct linked to the 3'UTR of each target gene shows repression with increasing expression of miR-499. Mutation of the sites blocks repression. (H) Western blot showing expression of beta-catenin in response to exercise. Beta-tubulin was used as a control for protein loading. (I-J) Western blot for beta–catenin under sedentary conditions and after 14 days of exercise in WT and TG mice, as well as WT versus KO animals. (K) Real Time RT-PCR analysis for known target genes of beta-catenin in hearts of sedentary (Sed) mice and after 14 days of exercise training (Ex). (L-N) Real Time RT-PCR for Igf-1 and Egfr transcripts after exercise comparing wild type versus TG, WT and KO and antimiR-control versus antimiR-499. (O) VW/BW after 2 weeks of exercise training between heterozygous beta catenin floxed mice in the absence and presence of αmhc-Cre transgene.*$p<0.05$

The Fzd4 and Lin7c mRNAs each contain a single conserved binding site for miR-499 (Figure 2.10 E). miR-499 dose-dependently repressed expression of a luciferase reporter linked to the Fzd4 and Lin7c 3' UTRs, and mutation of nucleotides 2 and 3 of the miRNA binding site abolished the repression by miR-499 (Figure 2.10 F-G). These results suggest that miR-499 directly represses the expression of Fzd4 and Lin7c.

Beta-catenin has been suggested to be modulated during physiological hypertrophy through IGF-1/Akt signaling (Aschenbach et al., 2006; Sakamoto et al., 2004). However, the role of beta-catenin in exercise-induced cardiac hypertrophy has not been investigated. We therefore tested whether beta-catenin signaling regulates cardiac hypertrophy in response to exercise training. Using western blot analysis, we examined beta-catenin protein levels under sedentary

conditions and after 14 days of voluntary exercise training (n=3 per group) and found beta-catenin was markedly increased (~5 fold) after exercise training (Figure 2.10 H). Beta-catenin was down-regulated by miR-499 over-expression and increased in miR-499KO mice after exercise (Figure 2.10 I-J). We also examined the expression of genes previously identified to be positively regulated by Wnt/beta-catenin signaling (Du et al., 2009; Longo et al., 2002; Malliri et al., 2006; Shtutman et al., 1999; Tan et al., 2005). Two genes, Igf-1 and Egfr, were both modestly, but significantly, increased in response to exercise training (Figure 2.10 K) and were repressed by miR-499 over-expression (~2 fold) and increased (~2 fold) by genetic and antimiR mediated loss of miR-499 (Figure 2.10 L-N).

Finally, we tested the ability of beta-catenin to directly modulate exercise-induced cardiac growth. Previous studies demonstrated that conditional deletion of one allele of beta-catenin specifically in the heart was sufficient to blunt pathological remodeling (Qu et al., 2007). Using this model system, beta-catenin floxed/WT and beta-catenin floxed/WT, αMHC-cre positive mice were subjected to 14 days of voluntary exercise. As expected, the αMHC-cre negative mice underwent physiological hypertrophy as measured by a significant increase in VW/BW (Figure 2.10 O). However, mice lacking a single allele of beta-catenin showed reduced physiological cardiac hypertrophy following exercise training. These results suggest that exercise induced physiological hypertrophy is mediated

in part by beta-catenin expression and that miR-499 influences the stability of beta-catenin.

Discussion

The results of this study reveal a novel role for miR-499 in the regulation of cardiomyocyte growth in response to exercise. miR-499 expression levels are reduced in the heart by exercise training. Forced over-expression of miR-499 in the heart blunts exercise-induced cardiac growth, whereas genetic deletion of miR-499 increases physiological cardiomyocyte hypertrophy. Furthermore, the expression of miR-499 is inversely correlated to the exercise-induced activation of not only the Akt signaling pathway, but also to the stabilization of beta-catenin, which we demonstrate to be an important modulator of exercise-induced cardiac growth. Two miR-499 target genes that directly influence Akt signaling, p85α and Rictor, are reduced by overexpression of miR-499 and increased by loss of miR-499. Fzd4 and Lin7c, which stimulate beta-catenin signaling, are also induced in the heart by exercise training and are directly modulated by miR-499. These findings suggest that miR-499 functions as a key regulator of physiological remodeling of the heart, as schematized in Figure 2.11.

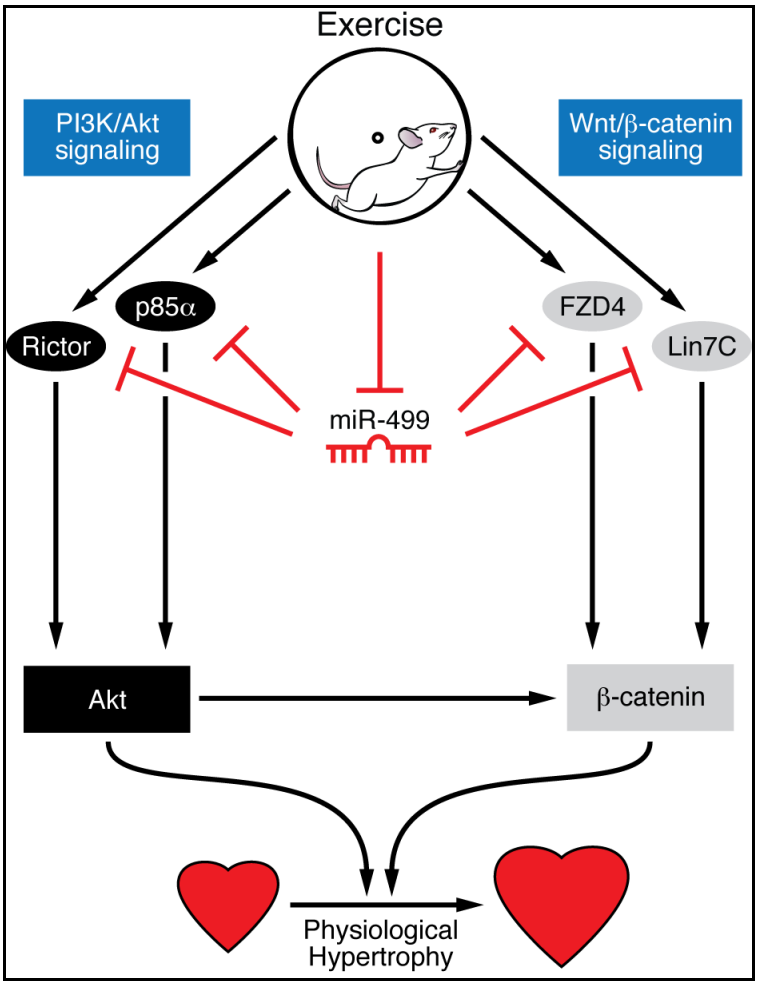

Figure 2.11: Model of regulation of physiological hypertrophy by miR-499. Proposed mechanism for miR-499 action in the heart during exercise induced cardiac hypertrophy. Exercise training up regulates p85α and Rictor in the PI3K signaling pathway and Fzd4 and Lin7c in the beta-catenin signaling pathway. Exercise represses the expression of miR-499 that directly represses p85α, Rictor, Fzd4 and Lin7c. Leading to increased Akt and beta-catenin activation and subsequently more physiological cardiac hypertrophy.

MiRNAs have been implicated in pathological cardiac remodeling, but their role in physiological remodeling has been largely unexplored. Mounting

evidence suggests that distinct signaling pathways and gene regulatory programs mediate physiological and pathological cardiac growth responses (Dorn and Force, 2005; Hill and Olson, 2008). However, how large gene networks are regulated in response to exercise training is not understood. MiRNAs are known to influence the expression of large numbers of target transcripts, such that a single miRNA can affect the expression of hundreds of potential high- and low-affinity target genes (Small and Olson, 2011). The current study suggests that exercise-induced cardiac hypertrophy is associated with a unique miRNA expression signature in the heart. These findings imply that miRNAs are an important component of a regulatory network of genes that mediates the physiological cardiac remodeling program.

The role of miR-499 as a regulator of exercise-induced cardiac remodeling is of particular interest in light of previous studies examining miR-499 in pathological cardiac remodeling(Shieh et al., 2011; Wang et al., 2011). miR-499 was originally identified as a member of a group of miRNAs, including miR-208a, miR-208b and miR-499, which are encoded within the introns of Myh6, Myh7 and Myh7b myosin genes, respectively(van Rooij et al., 2009). miR-499 was shown to be required for re-expression of Myh7, a hallmark of the fetal gene program, following propylthiouracil (PTU) treatment(van Rooij et al., 2009). Recent work demonstrated that robust over-expression of miR-499 (~50-fold) leads to cardiomyocyte hypertrophy, apoptosis, fibrosis and contractile

dysfunction (Shieh et al., 2011). Thus, miR-499 appears to be both necessary and sufficient for pathological cardiac remodeling. However, the cardiac expression levels of miR-499 in the transgenic mouse model used in the current study were much lower (~3-fold) and these mice did not display any overt signs of cardiac dysfunction. Interestingly, Shieh et al. also reported that mice over-expressing miR-499 at lower levels had no baseline cardiac phenotype (Shieh et al., 2011). These findings suggest that the pathological effects of miR-499 over-expression are dose-sensitive.

Recent findings using transgenic mice expressing dominant negative and constitutively active PI3K/Akt imply that there is considerable cross-talk between physiological and pathological signaling pathways. Overexpression of constitutively active PI3K was capable of blunting pathological remodeling in a mouse model of dilated cardiomyopathy (McMullen et al., 2007). The possibility that hyper-activation of the PI3K/Akt signaling pathway in miR-499 knockout mice may contribute to the inhibition of pathological growth following TAB warrants future investigation.

The IGF/PI3K/Akt growth axis has been described as a canonical signaling pathway mediating physiological growth of the heart (Balasubramanian et al., 2009; DeBosch et al., 2006; Dorn and Force, 2005; Lin et al., 2010). The expression levels of miR-499 were inversely related to the activation status of the PI3K/Akt signaling pathway following exercise. Furthermore, miR-499 gain- and

loss-of-function were associated with repression and activation of PI3K/Akt, respectively. We have identified two miR-499 target genes, p85α and Rictor, both of which are known to positively regulate PI3K/Akt signaling. p85α is one of three regulatory subunits that form complexes with the p110 catalytic subunits of PI3K, which are engaged by growth factor receptors upon ligand stimulation. In response to growth factor signaling initiated by exercise, p85α and p85β have been demonstrated to be required for phosphorylation of Akt and necessary for physiological cardiac hypertrophy (Luo et al., 2005). Rictor is a member of the mTORC2 signaling complex, which is activated by PI3K/Akt signaling following growth factor stimulation and promotes Akt phosphorylation (Balasubramanian et al., 2009). Therefore, we describe two separate components of the exercise-induced Akt signaling cascade that are directly modulated by the expression of miR-499.

It has been previously shown that activation of PI3K/Akt signaling in muscle leads to the stabilization of beta-catenin (Aschenbach et al., 2006). However, the role of beta-catenin in exercise-induced cardiac hypertrophy has not been previously studied. Interestingly, unbiased analyses of miR-499 target gene regulation identified a number of positive regulators of beta-catenin stability. We therefore explored the role of beta-catenin in physiological cardiac hypertrophy and found that beta-catenin is stabilized in the heart in response to exercise training and that haploinsufficiency of beta-catenin blunts the exercise-induced

hypertrophic response. Beta-catenin levels were profoundly affected by miR-499 expression following exercise. We were able to confirm that miR-499 directly represses Fzd4 and Lin7c both known regulators of beta-catenin stabilization, during exercise. Fzd4 is a receptor that mediates both canonical- and non-canonical- Wnt signaling depending on its signaling ligand, the canonical Wnt signaling leads to transcriptional activation of canonical beta-catenin signaling (Zhang et al., 2011). In contrast, Lin7c is a scaffolding protein, which has been shown to increase the levels beta-catenin (Onda et al., 2007). Thus, miR-499 appears to control physiological cardiac growth through regulation of multiple components of the PI3K/Akt and beta-catenin signaling pathways. Although our findings reveal a previously unappreciated role for miR-499 in the regulation of exercise-induced cardiac hypertrophy, we cannot formally exclude a possible contribution of miR-499 in skeletal muscle to the observed phenotypes.

Interestingly, our results found no differences under sedentary conditions in either cardiac size or baseline regulation of Akt and beta-catenin signaling pathways. These findings suggest miR-499 is not required for physiological growth during development or alternatively compensatory mechanisms are involved during development which allow the heart grow normally during the early developmental window. Our findings are consistent with a number of previous reports that have demonstrated the role of miRNA in a particular process

is not fully appreciated until a stress condition in applied to the system (Leung and Sharp, 2010).

In conclusion, our results demonstrate that miR-499 is down-regulated in the heart in response to exercise training and that this repression is required for physiological cardiac remodeling. Genetic loss of function and antimiR mediated repression of miR-499 allows for increased physiological hypertrophy, whereas modest overexpression of miR-499 blunts cardiac growth following exercise. We provide evidence that miR-499 regulates myocyte hypertrophy through regulation of both PI3K/Akt and beta-catenin signaling. These results demonstrate that miR-499 is an integral regulator of a large gene network controlling exercise-induced cardiac hypertrophy.

Methods

Exercise Protocol and Tissue Collection

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Adult male mice were housed individually and given access to a voluntary running wheel. Wheel rotation was electronically monitored and the number of revolutions were recorded at 5 minute intervals. Animals were given three days to acclimate to the voluntary exercise regime before measurements were recorded. Sedentary animals were age matched, littermate controls and were housed communally. Total distance exercised was measured by multiplying the total number of revolutions

over the training period by the circumference of the wheel which was 0.357 meters. For heart weight and body weight measurements, animals were euthanized by CO_2 asphyxiation. Body weight was measured and hearts were excised, rinsed in PBS and the atrias removed, blotted and weighed. The ventricles were snap-frozen in liquid nitrogen and stored at -80˚C.

Microarray of miRNAs

miRNA profiling of heart ventricles from sedentary mice versus 10 days of voluntary exercise training (adult C57BL/6 mice; ($n \geq 5$)) was performed using the LC Science's miRNA microarray service, (Houston, TX). Details of sample preparation and analysis are as previously described (van Rooij et al., 2008).

RNA Analysis

Total RNA was extracted from mouse ventricles using TRIzol (Invitrogen, CA) according to manufacturer's protocol. Quantitative real-time PCR was performed using an ABI 7000 cycler. cDNA was produced by reverse transcription using random hexamers (Invitrogen, CA). For detection of miRNA expression levels by real-time PCR, RT-PCR was performed using Taqman microRNA Reverse Transcriptase kit (Applied Biostystems, CA) and TaqMan probes (Applied Biosystems, CA), according to the manufacturer's recommended protocol. Taqman chemistry (Applied Biosystems, CA) was used for analysis of [miR-499, miR-214, miR-29a, miR-29c, miR-30a, miR-30e], Myh7b, Fzd4 and p85α (mRNA ABI assay ID listed in Table 2.2). SYBR green chemistry was

employed for analysis of Egfr, Igf-1, Lin7C and Rictor mRNA. 18S ribosomal RNA was used as a housekeeping control. Primers are listed in Table 2.3.

For detection of miRNA by Northern blot, 10 µg of total RNA was loaded into each lane. RNA samples were run on a 20% acrylamide denaturing gel and transferred to Zeta-probe GT genomic blotting membrane (Bio-Rad, CA) by electrophoresis. After transfer, membranes were UV crossed-linked. A probe for miR-499 detection was labeled with γ-^{32}P-ATP using T4 polynucleotide kinase (New England Biolabs, MA). Probes were hybridized to the membranes overnight at 39˚C in Rapid-hyb buffer (GE Healthcare). The following day, membranes were washed twice with 0.5x SSC containing 1% SDS at 39˚C for 15 min. Blots were exposed and analyzed by PhosphoImager (GE HealthCare Life Sciences). A U6 probe served as a loading control for all blots.

In Vivo Delivery of LNA-antimiR Oligonucleotides to Adult Mice

Locked nucleic acid (LNA) modified antimiR directed against the mature miR-499 sequence were synthesized as unconjugated and fully phosphorothiolated oligonucleotides (miRagen Therapeutics, CO). The LNA-antimiR was designed to complement 16 nucleotides of the mature miR-499. A control LNA-antimiR was used, which contained the same LNA/DNA chemical composition as the antimiR-499 oligo, but was designed to target a miR sequence expressed in *C. elegans* but not expressed in mammals. Adult mice (10 weeks of age C57BL/6, The Jackson Laboratory) received control oligo or antimiR-499 at a

dose of 10mg/kg body weight via tail vein injection once per day for three consecutive days. Hearts were harvested two days after treatment and after 14 days of exercise training for assessment of miRNA knockdown, histological analysis and heart weight/body weight measurements.

Histological Analysis

Hearts used for histology were briefly rinsed in PBS, fixed in 4% paraformaldehyde overnight, and then transferred to 50% ethanol until paraffin embedding. Sections (5 μm thickness) were processed for H&E according to standard procedures.

Immunofluorescence

All immunofluorescence was performed on paraffin sections as previously described (Porrello et al.). The following primary antibodies were used: phospho-histone H3 (Ser10) (Millipore, MA) at a dilution of 1:100 and wheat germ agglutinin conjugated to Alexa Fluor 594 (50 μg/ml, Invitrogen, CA). Anti-rabbit secondary antibody conjugated to Alexa Fluor 555 at a dilution of 1:400 (Invitrogen, CA). Nuclei were identified by counter-staining sections with Hoechst 33342 (Invitrogen, CA).

For detection of apoptotic nuclei, paraffin sections were processed for immunofluorescent TUNEL staining using the *In Situ* Cell death Detection KIT TMR Red (Roche Applied Sciences, IN) according to the manufacturer's instructions.

Protein Analysis/Western Blotting

Total heart ventricle lysates were prepared by homogenizing tissue in RIPA lysis buffer (50mM Tris-HCL pH 7.4, 150mM NaCl, 1mM EDTA, 1%Triton X-100, 0.1% SDS, 1% sodium deoxycholate) and were resolved by SDS-PAGE and analyzed to detect Akt1, phospho-Akt1, beta-catenin and tubulin using 20 μg of protein per sample. The proteins were transferred to PVDF membrane (Millipore, MA). Santa Cruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology, CA) was used for detection following the manufacturer's recommended instructions. Primary antibodies used include rabbit polyclonal Akt1 (#9272, Cell Signaling, MA) at a dilution of 1:1000, rabbit polyclonal phospho-Akt1 Serine-473 (#4058, Cell Signaling, MA) at a dilution of 1:1000, mouse monoclonal beta-catenin (SC-7963, Santa Cruz Biotechnology, CA) at a dilution of 1:50 and mouse monoclonal tubulin at a dilution of 1:5000 (T9026, Sigma-Aldrich, MO). HRP-conjugated secondary antibodies were used according the manufacturer's recommended instructions (Biorad, CA).

Cell Culture, Transfection and Luciferase Assays

Approximately 500-bp genomic fragments of Fzd4, Lin7c, P85α and Rictor encompassing the miR-499 binding site were amplified by PCR (primers listed in Supplemental Figure 4) and cloned into the firefly luciferase reporter

construct (pMIR-ReprotTM; Ambion, TX). The seed region of the miR-499 target sites was mutated with QuickChange II site-directed mutagenesis kit (Stratagene, CA). Primer sequences are provided in Supplemental Table 4. Luciferase assays were carried out in HEK293 cells and transfected with Fugene6 (Roche Applied Science, IN).

Transthoracic Echocardiography

Cardiac function was evaluated by two-dimensional echocardiography using a Visual Sonics Vevo 770 Ultrasound (Visual Sonics, Canada) and a 30-MHz linear transducer. M-mode tracing was used to measure anterior and posterior wall thickness at end diastole and end systole. Left ventricular (LV) internal diameter (LVID) was measured as the largest anteroposterior diameter in either diastole (LVIDd) or systole (LVIDs). LV fractional shortening (FS) was calculated according to the following formula: FS(%) = [(LVIDd-LVIDs)/LVIDd] x100.

Statistics

We evaluated statistical significance by 2-tailed Student's *t* test, with $p \leq 0.05$ regarded as significant. We show all data as mean ± SEM.

Gene	NCBI Reference Sequence	Assay ID
Fzd4	NM_008055.4	Mm00433382_m1
P85α (Pik3r1)	NM_011625.1	Mm01339719_m1
Myh7b	NM_001085378.1	Mm01249941_m1
Gene	**miRBase Accession Number**	**Assay ID**
miR-499	MIMAT0003482	001352
miR-29a	MIMAT0000535	002112
miR-29c	MIMAT0000536	000587
miR-30a	MIMAT0000128	000417
miR-30e	MIMAT0000248	002223
miR-214	MIMAT0000661	002306

Table 2.2: List of Applied Biosystems Assay ID for real-time PCR analysis.

Gene	NCBI Reference Sequence	Primer Sequences
Lin7C	NM_011699.3	F 5'-ATCCAAGGCCCTCTTCTGTT-3' R 5'-AGTGAGAGCCAATGCAACTG-3'
Rictor	NM_030168	F 5'-GATGGCCCAGCTTTCTCATA-3' R 5'-GAACGTCCCGCTCGATCT-3'
Igf-1	NM_184052	F 5'-CACTCATCCACAATGCCTGT-3' R 5'-ATCCTCTGCAGGCTCAGAAA-3'
Egfr	NM_207655.2	F 5'-TGGATGCTCTTCAGTTCGTG -3' R 5'-GGCGTTGGAGGAAAAGAAAG-3'

Table 2.3: List of primer sequences for SYBR real-time PCR analysis

Chapter III

Conclusions and Future Remarks

Heart disease is the leading cause of morbidity and mortality in the western world. To that end, scientists over the last half century have used numerous biochemical and genetic based approaches to begin to understand the causes and molecular machinery involved in detrimental and beneficial remodeling of the heart. Recent research on miRNAs has identified these genes as key regulators of pathological cardiac remodeling and may provide a new possibility for therapeutic interventions. Here we identify miRNAs, specifically miR-499, as key regulators of beneficial exercise-induced cardiac remodeling and may also provide useful as a preventative therapeutic. However several important questions remain.

miR-499 as a regulator of exercise-induced cardiac hypertrophy

This study has determined the expression of miR-499 in the heart is repressed by exercise training, and modest re-expression of miR-499 is capable of blunting physiological cardiac growth. In contrast, genetic and acute repression of miR-499 allows enhanced cardiac growth after exercise training. Cardiac function was preserved in both gain- and loss- of function groups. However, the experiments were performed over a short period of time and it is unclear if long-term exercise training may affect these results. Over the short period of time, the observed growth may be compensatory hypertrophy but may eventually become decompensated remodeling and result in heart failure. In contrast, long-term

treatment could also lead to an eventual increase in cardiac function. Additionally, long-term analysis would give insight into whether there is growth ceiling to exercise-induced cardiac remodeling.

Regulation of Akt and Beta-catenin by miR-499

PI3K/Akt signaling has previously been identified by many groups as a key regulator of excised-induced cardiac growth (Bernardo et al., 2010). In the current study, we have demonstrated miR-499 acts as novel regulator of PI3K/Akt signaling using biochemical analysis and identified two known members of the PI3K/Akt signaling pathway as direct miR-499 target genes. However, since miRNAs are known to have multiple targets it is possible this research is only scratching the surface as to how miR-499 regulates PI3K/Akt signaling. The regulation of beta-catenin stabilization by miR-499 is clearly robust but once again it is unclear how important the direct repression of Fzd4 and Lin7c are in the total stabilization of beta-catenin. Finally, no definitive studies have tested *in vivo* the function of Rictor, Fzd4 or Lin7c in the context of exercise-induced cardiac hypertrophy. Further genetic experiments *in vivo* would elucidate the relative contribution of each gene.

Therapeutic Implications of antimiR-499

The search for a therapeutic to prevent cardiac injury or enhance function after cardiac insult has been a long term goal. Inhibition of miR-499 by antimiR-499 may be a potential tool to accomplish this goal. Our results have demonstrated pre-treatment of mice with antimiR-499 followed by voluntary exercise training results in increased physiological hypertrophy perhaps "an athlete's heart". It has been suggested elite endurance athletes have a better prognosis after cardiac injury, (Hill and Olson, 2008) therefore if antimiR-499 works in a similar manner in humans as it does in rodents it could be useful as a preventative therapeutic for heart disease. In contrast, animal and human experiments have demonstrated after myocardial infarction that exercise training can improve cardiac performance. It would of interest to investigate if exercise and antimiR treatment following cardiac insult lead to greater improvements in cardiac function.

In conclusion, our results demonstrate miRNAs are not only regulated during exercise-induced cardiac hypertrophy but in the case of miR-499 also play key roles in the regulation of signaling cascades associated with this type of remodeling. Future experiments will elucidate the potential importance of other miRNAs identified in this study for exercised-induced cardiac remodeling. Further experiments may also unravel the question of therapeutic regulation of miRNAs for enhanced cardiac function is capable of preventing heart disease.

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