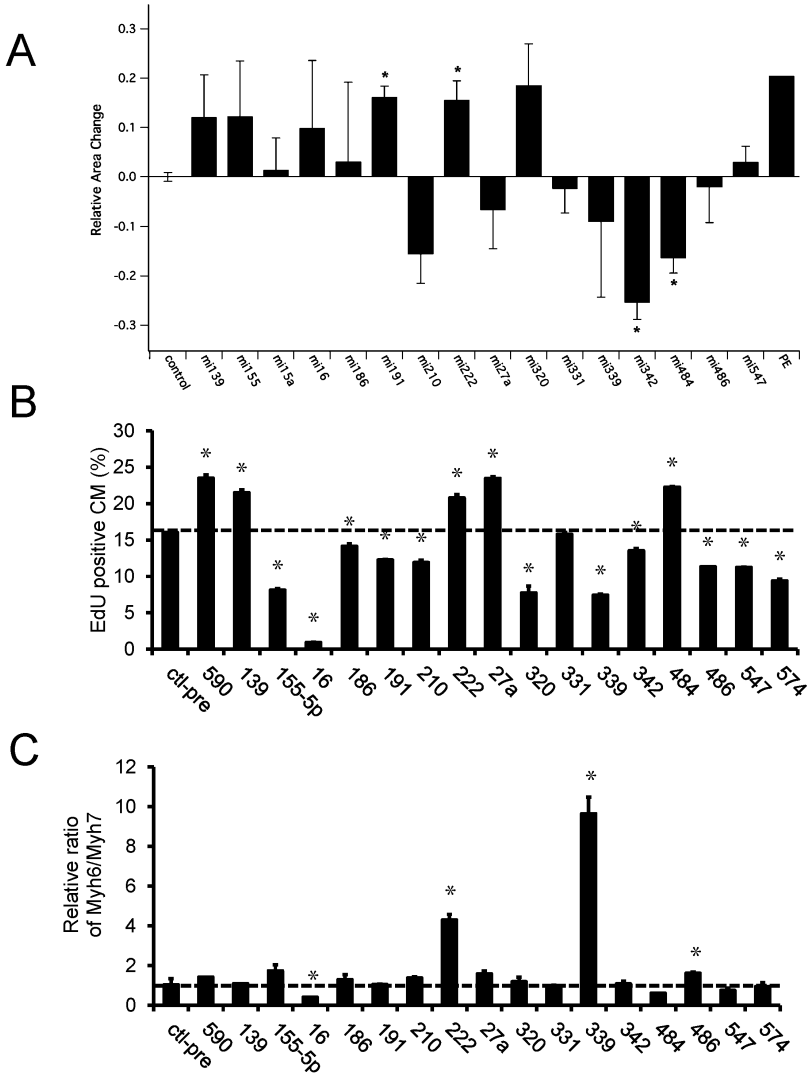

Figure S1 related to Figure 1. Functional effects of validated microRNAs in neonatal cardiomyocytes

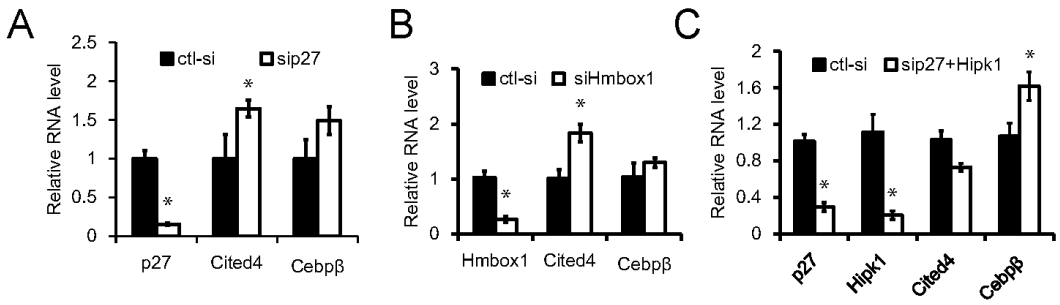

Figure S2 related to Figure 3. miR-222 targets affect CITED4 and C/EBPβ in neonatal cardiomyocytes

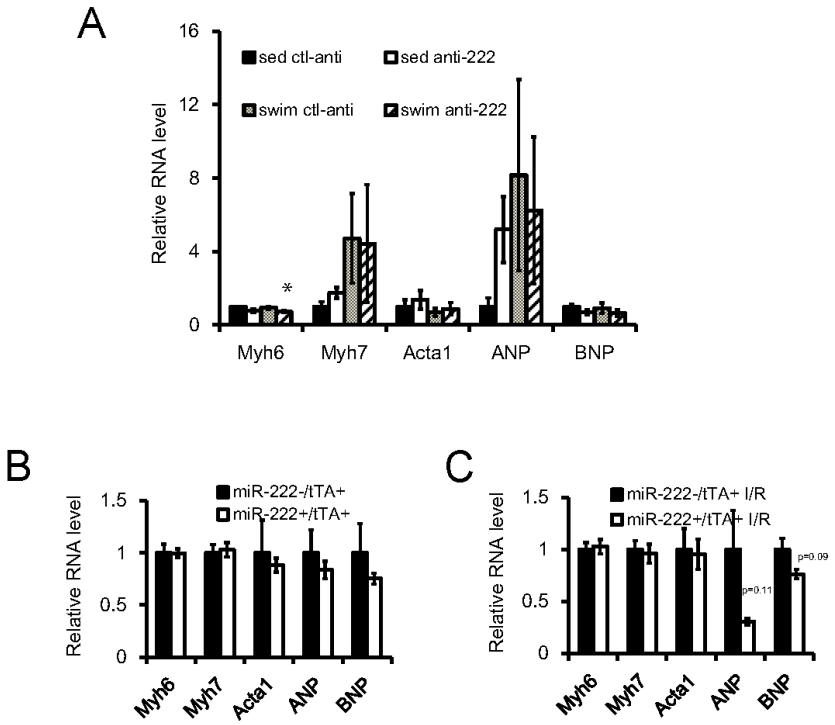

Figure S3 related to Figures 4 and 5. Cardiac hypertrophic markers in mice injected with LNA-anti-miR-222 and Tg-miR-222 mice

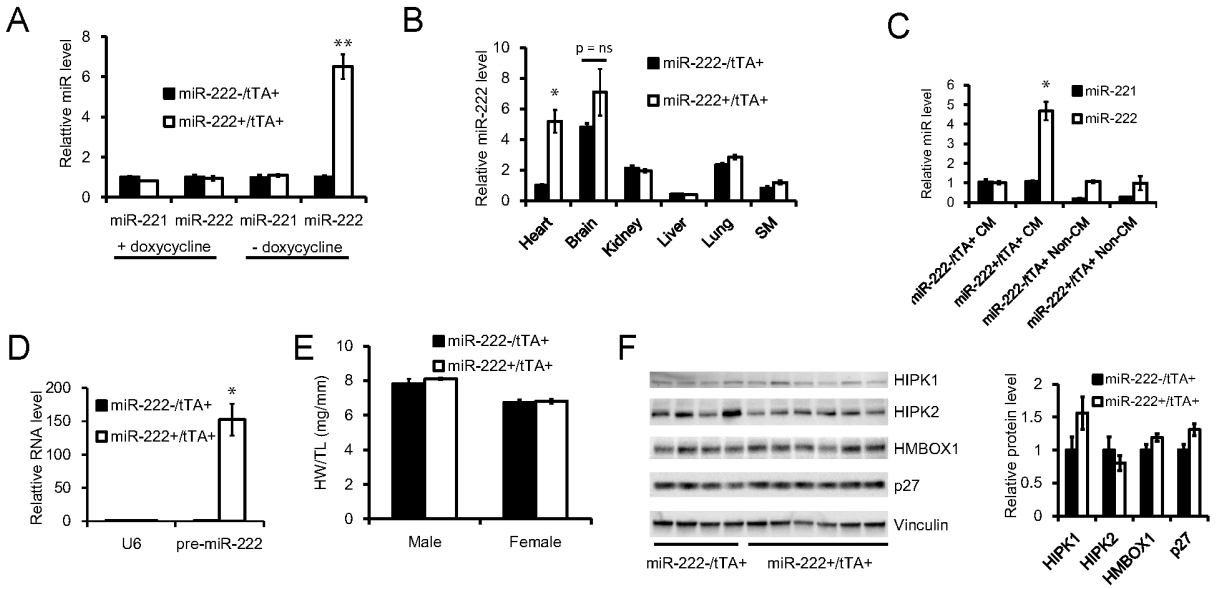

Figure S4 related to Figure 5. Baseline characteristics of Tg-miR-222 mice

Legends of Figures S1-S4

Figure S1 related to Figure 1. Functional effects of validated microRNAs in neonatal cardiomyocytes

A. Immunohistochemistry against sarcomeric α-actinin followed by cell area quantifications in neonate cardiomyocytes transfected with the indicated microRNAs or treated with phenylephrine (PE). **B**. Flow cytometry analysis of EdU incorporation in neonate cardiomyocytes transfected with control precursor, precursors of indicated miRNAs, or precursor for has-miR-590-3p as a positive control. Cumulative data from three independent experiments are shown. **C**. qRT-PCR analysis of the relative ratio of α/β-MHC in neonate cardiomyocytes transfected with indicated miR precursors.

Cumulative data from three independent experiments are shown. All data are represented as mean ± SEM. *p<0.05 versus respective control using Student's test.

Figure S2 related to Figure 3. miR-222 targets affect CITED4 and C/EBPβ in neonatal cardiomyocytes

A-C. qRT-PCR analysis of the levels of CITED4 and C/EBPβ in neonatal cardiomyocytes transfected with control siRNA or siRNA for p27 (A), Hmbox1 (B), add p27 plus Hipk1 (C). Cumulative data from three independent experiments are shown. Error bars stand for standard errors. *p<0.05 versus control using Student's test.

Figure S3 related to Figures 4-5. Cardiac hypertrophic markers in mice injected with LNA-anti-miR-222 and Tg-miR-222 mice

A. qRT-PCR analysis of hypertrophic markers in hearts of sedentary and swum mice injected with control LNA-antimiR (sed ctl-atni, n=5 and swim ctl-anti, n=5) or LNA-antimiR-222 (sed anti-222, n=5 and swim anti-222, n=5). **B**. qRT-PCR analysis of hypertrophic markers in hearts at baseline after miR-222 induction for 4 weeks. Cumulative data from 4 mice for miR-222-/tTA+ and 9 for miR-222-/tTA+ are shown. **C**. qRT-PCR analysis of hypertrophic markers in miR-222-expressing hearts 1 week after ischemic injury. Cumulative data from 4 mice for each genotype are shown. Error bars stand for standard errors. *p<0.05, versus sed ctl-anti using One-way ANOVA.

Figure S4 related to Figure 5. Baseline characteristics of Tg-miR-222 mice

A. qRT-PCR analysis of microRNA expression in hearts at baseline after miR-222 induction for 4 weeks. Data are shown as fold induction of miR expression normalized to U6. Cumulative data from 4 to 6 mice for each genotype are shown. **B** and **C**. qRT-PCR analysis of miR expression in tissues (B), isolated adult cardiomyocytes and non-cardiomyocytes (C) from hearts of transgenic mice at baseline after miR-222 induction for 4 weeks (n=4 hearts in each group). **D.** qRT-PCR analysis of pre-miR-222 in hearts at baseline after miR-222 induction for 4 weeks (n=4 hearts in each group). **E**. Heart weight/tibial length (HW/TL) ratios of transgenic mice (male and female) at baseline after miR-222 induction for 4 weeks (n=5~8 hearts in each group). p=ns. **F.** Western blot results show no significantly different expression of HIPK1, HIPK2, HMBOX1 and p27 in miR-222-expressing hearts at baseline after miR-222 induction for 4 weeks (miR-222-/tTA+, n=4; miR-222+/tTA+, n=6). Error bars stand for standard errors. *p<0.05, **p<0.01 compared with respective controls using Student's test.

Table S1 related to Figure 1. Differentially regulated microRNAs in hearts of 2 exercise models.

Table S2 related to Figures 1-3. List of primers used in qRT-PCR.

Table S3 related to Figures 3 and 5 . List of primers used in for constructs.

Primer name	Sequence (5'-3')
miR-222F	ATATGTCGACAGGAAGTGAATCTAAAGGTAGTTAAG
miR-222R	GCACAAGCTTCTGAATGAATGAATAATGGAAAATTAGATG
mHmbox1_3'F	ATATCTCGAGTTGGAATTACCTTGGAAGTT
mHmbox1_3'R	GAACGCGGCCGCAGTGACAGTGTGCTATTTAATC
mHipk1_3'F	ATATCTCGAGAAGAAGAACCCCATTTCAAA
mHipk1_3'R	GAACGCGGCCGCACAACGAATCCAAAGATAAACTA
mHipk2_3'F1	ATATCTCGAGGTTATTCCTCAGATGTCATTA
mHipk2_3'R1	GAACGCGGCCGCAACATAAAGACCCAAGTAAA
mHmbox1_3'MF	GGAATTTTTTTCCTTGATCATATGCACTTTTGTTACTTTTTAAAG
mHmbox1_3'MR	CTTTAAAAAGTAACAAAAGTGCATATGATCAAGGAAAAAAATTCC
mHipk1_3'MF	CAGCTTGAAAAATAATCTCACTATGCAGTACATTATATGTAC
mHipk1_3'MR	GTACATATAATGTACTGCATAGTGAGATTATTTTTCAAGCTG
mHipk2_3'MF1	GGTTGTCCTGGACACCCTAGAAGAGCACTGCACCCTTGTTTCC
mHipk2_3'MR1	GGAAACAAGGGTGCAGTGCTCTTCTAGGGTGTCCAGGACAACC

Table S4 related to Figure 5. Echocardiography analyses of cardiac function of mice 4 weeks after doxycycline removal

	miR-222-/tTA+ (M, n=10)	miR-222+/tTA+ (M, n=10)	miR-222-/tTA+ (F, n=10)	miR-222+/tTA+ (F, n=11)
IVSd (mm)	0.71±0.06	0.69±0.08	0.70±0.06	0.70±0.05
IVSs (mm)	1.29±0.09	1.29±0.11	1.24±0.10	1.25±0.06
LVIDd (mm)	3.43±0.06	3.28±0.07	3.12±0.08	2.96±0.06
LVIDs (mm)	1.62±0.07	1.5±0.07	1.50±0.06	1.42±0.04
LVPWd (mm)	1.08±0.04	1.15±0.03	1.12±0.02	1.16±0.03
LVPWs (mm)	1.66±0.06	1.78±0.03	1.67±0.04	1.65±0.03
%FS (mm)	53.1±1.40	54.57±1.57	51.85±1.39	52.14±0.95
HR (BPM)	693.15±8.28	699.87±6.42	692.37±10.96	706.16±9.41

IVSs: systolic interventricular septum; IVSd diastolic interventricular septum; LVIDd: left ventricular end diastolic internal dimension; LVIDs: left ventricular end systolic dimension; LVPWd: left ventricular end diastolic posterior wall; LVPWs: left ventricular end systolic posterior wall dimension; FS (%): fractional shortening

Supplemental Experimental Procedures

Materials

Taqman Rodent miRNA microarray cards and assays, TaqMan® MicroRNA Reverse Transcription Kit, TaqMan® Universal Master Mix II, no UNG, Ambion® Pre-miR™ miRNA Precursors, siRNA, Lipofectamine RNAiMAX, Opti-MEM® I Reduced Serum Media, High-Capacity cDNA Reverse Transcription Kit, Power SYBR® Green PCR Master Mix, Click-iT EdU Flow Cytometry Assay, Click-iT® EdU Alexa Fluor® 555 Imaging Kits, ProLong® Gold Antifade Reagent with DAPI, APO-BrdU TUNEL assay, and Click-IT TUNEL Alexa Flour 594 image assay were purchased from Life technologies.. iQ SYBR Green Supermix was ordered from BIO-RAD. Luciferase reporter system was purchased from Promega. A list of utilized primers is presented in Table S2. *In vivo* LNA microRNA inhibitors were purchased from Exiqon.

RNA Isolation, Quantitative Real Time PCR (qRT-PCR) and Microarray

Methods were used as described before (Liu et al., 2009). Briefly, RNAs from cultured cells and tissues were isolated TRIzol (Invitrogen) following the manufactures' manual. qRT-PCR for microRNA was performed on cDNA generated from 100 ng of total RNA using the protocol of the TaqMan microRNA assays protocol (Invitrogen). qRT-PCR for regular genes was performed on cDNA generated from 200 ng of total RNA using the protocol of a high capacity cDNA reverse transcription kit (Invitrogen). Amplification and detection of specific products were performed on a Biorad CFX384 qPCR System. U6 or sno202 as an internal control was used for microRNA template normalization and GADPH or U6 was used for other template normalization. The sequences of the primers used are listed in Table 3. The relative gene expression was

calculated by comparing cycle times (Ct) for each target PCR as previously described (Liu et al., 2009). Taqman-based miRNA microarray and individual Taqman-based miRNA assays were carried out following standard procedures.

For checking micorRNA level in human serum, total RNA was isolated from a 400 μL aliquot of serum using a mirVana PARIS isolation kit (Ambion, Austin, Texas) according to the manufacturer's instructions for serum samples without enrichment for small RNAs. The *Caenorhabditis elegans* miRNA (cel-miR-39) of 50 pmol/L was added as the spike-in control after the equal volume of denaturing solution was added. For quantitative miRNA analysis, the Bulge-LoopTM miRNA qPCR Primer Set (RiboBio) was used to detect miR-222 expression by qRT-PCRs with SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA) using the 7900HT Fast Real-Time PCR System on 10 μL of PCR master mix containing 1 μL of Forward primer, 1 μL of Reverse primer, 5 μL SYBR Green (2×), 2 μL of RT products, and 1 μL of ddH2O. The cycling parameters were as follows: denaturation at 95 °C for 15 s; annealing at 60 °C for 30 s; and elongation at 72 °C for 30 s (40 cycles) with an initial cycle of 95°C for 3 min. Cel-miR-39, which lacks sequence homology to human miRNAs was used for normalization. The qRT-PCR reactions were run in triplicate, and the signal was collected at the end of every cycle.

Western Blot, Immunochemistry and Immunofluorescence Staining

Proteins were isolated from cultured NRVMs and hearts and protein levels were determined by western blot analysis. Briefly, equal amounts of protein determined by BCA protein assay kit (Pierce) were subjected to SDS-PAGE. Standard western blot analysis was conducted using p27 (Kip1) (1:1000, Cell Signaling #3698), HIPK1 (1:500,

Abcam ab90103), HIPK2 (1:500, Cell Signaling #5091), and HMBOX1 (1:500, Abcam ab101140). HSP90 (1:1000, Cell Signaling #4874) or Vinculin (1:1000, Sigma V9264) was used as a loading control.

Hearts were snap frozen in OCT in liquid nitrogen and sectioned on short axis at 5 μm. Frozen sections were stained with wheat germ agglutinin to outline only exactly cross-sectional cardiomyocytes. Cells were labeled with 10 μM (Figure 2C) or 20 μM EdU (all other experiments) for 24 h before harvesting and Click-IT EdU 555 Imaging kit was used to reveal EdU incorporation. Click-IT TUNEL Alexa Flour 594 image assay was used to check cell apoptosis. To visualize fibrosis, Masson's Trichrome staining was performed by BIDMC histology core facility. For staining, sections were fixed in 4% paraformaldehyde (PFA) followed by washing in PBS. Sections were blocked with 3% (w/v) BSA in PBS and then incubated with primary antibodies applied at 1:100 dilution (unless otherwise indicated) for 1 hour at room temperature. Primary antibodies included: cardiac troponin-I (Abcam ab56357), cardiac troponin-T (Abcam ab10214), α-actinin (Sarcomeric, Sigma A7732), Ki67 (Cell Signaling #9129), and phospho-Histone H3 (Cell Signaling, #3377).

Luciferase Assays

A reporter plasmid was constructed by inserting a fragment of the 3'-UTR of *Hipk1*, *Hipk2* or *Hmbox1* mRNA containing the putative miR-222 binding site into a firefly luciferase reporter plasmid psiCHECK-2 (Promega). As a mutated control, the construct with mutated fragment of the 3'-UTR of *Hipk1*, *Hipk2* or *Hmbox1* mRNA without the putative miR-222 binding site was made by using quikchange site-directed mutagenesis kit (Agilent). The primers used for constructs and mutation are shown in

Table S3. COS7 cells were cotransfected with the reporter plasmid or the mutated construct (100 ng) and Ambion pre-miR miRNA scramble or miR-222 precursor (1 μM) according to the lipofectamine 2000 transfection procedures (Invitrogen). 48 hours after transfection, cells were lysed. With isolated cell lysates, relative luciferase expression was measured on a multi-mode multi-format reader SpectraMax M5 by using a dual luciferase reporter system (Promega).

Flow cytometry

One million isolated NRVMs were plated into a 6cm diameter BD Primaria tissue culture dish. Twenty-four hours after plating, cell were transfected with 20 μM LNA or 0.4 μM RNA oligo precursor, or 20 μM stealth siRNA via lipofectamine RNAiMax overnight. Then cells were synchronized for 24 hours in serum free media and then cultured in low serum media. Forty-eight hours after transfection, cells were labeled with 20μM EdU for 24 hours. Before harvesting, cells were incubated with 50 μM mitotracker orange for 45 minutes. Collected cells were stained by using the protocol of Click-iT EdU Flow Cytometry Assay (Invitrogen). To induce cell death, cells after treatments were switched to serum free media with/without addition of 0.3μM doxorubicin. Twenty four hour incubation, cells were harvested and stained by using the protocol of APO-BrdU TUNEL assay (Invitrogen). Stained cells were analyzed in a 5-laser LSR II machine in BIDMC flow core facility. At least 10,000 events were recorded by flow cytometry in each treatment. Flowjo7.6.1 was used to analyze flow data.

Supplemental References

Liu, X., Cheng, Y., Zhang, S., Lin, Y., Yang, J., and Zhang, C. (2009). A Necessary Role of miR-221 and miR-222 in Vascular Smooth Muscle Cell Proliferation and Neointimal Hyperplasia. Circulation Research *104*, 476-487.