SUPPLEMENTAL MATERIALS

METHODS

Generation of miR-21 mutant mice. To generate the miR-21 targeting vector, a 4.8 kb fragment (5' arm) extending upstream of the miR-21 coding region was digested with SacII and NotI and ligated into the pGKneoF2L2dta targeting plasmid upstream of the loxP sites and the Frt-flanked neomycin cassette. A 2.2 kb fragment (3' arm) was digested with SalI and HindIII and ligated into the vector between the neomycin resistance and Dta negative selection cassettes. Targeted ES-cells carrying the disrupted allele were identified by Southern blot analysis. Three miR-21 targeted ES clones were used for blastocyst injection. The resulting chimeric mice were bred to C57BL/6 mice to obtain germline transmission of the mutant allele. PCR primer sequences are as follows: 5prime-arm-Forward- 5'-TACTGTTCTTGGTGTGCCAGAAGA-3' ; 5prime-arm-Forward- 5'-AAAGCAAAGCAAACATCTCTGG-3'. 3prime-arm-Forward- 5'-GAGCCCTTATACC-3' ; 3prime-arm-Reverse- 5'-GCTCGGAGTTTGAC-3' ; KOarm-Forward- 5'-AAACCCTGCCTGAGCACCTCGT-3' ; KOarm-Reverse 5'-CAAGTCTCACAAGACATAAG-3'. Genotyping primer sequences are as follows: miR-21-straightKO-Forward- 5'-CCGGCTTTAACAGGTG-3' ; miR-21-straightKO-Reverse- 5'-GATACTGCTGCTGTTACCAAG-3'. miR-21-conditional-Forward- 5'-GCTTACTTCTCTCTGTGATTTCTGTG-3' ; miR-21-conditional-Reverse- 5'-GGTGGTACAGCCATGCGATGTCACGAC-3'.

Northern blot analysis. Total RNA was isolated from mouse tissue samples by using Trizol reagent (Gibco/BRL). Northern blots to detect microRNAs were performed as

previously described (3). A U6 probe served as a loading control (U6 forward: 5-GTGCTCGCTTCGGCAGC-3, U6 reverse: 5-AAAATATGGAACGCTTCACGAATTTGCG-3). Northern blot analysis for the experiments for which antimiR-21 was used were electrophoresed on non-denaturing gels to show the heteroduplex formation between the antimiR-21 and mature miR-21. Briefly, 12 ug of total heart RNA was loaded unheated on a native 20% acrylamide gel, ran for 1.5 hours at 150V, and transferred to a Zeta-probe blotting membrane (Bio-rad) for 2 hours at 90V. Membranes were probed with a ^{32}P-labelled LNA-modified oligonucleotide probe for miR-21 (Exiqon) and hybridized overnight at 37°C using RapidHyb buffer (GE Healthcare). Blots were visualized using a Storm 860 scanner (Molecular Dynamics).

RT-PCR and Real-time RT-PCR analysis. Total RNA from cardiac tissue was isolated using Trizol (Invitrogen). RT-PCR with random hexamer primers (Invitrogen) was performed on RNA samples, after which the expression of a subset of genes was analyzed by either a regular or quantitative real-time RT-PCR using gene specific primers or Taqman probes purchased from ABI. Primers to detect TMEM49 transcript are as follows: TMEM49-Exon10-Forward- 5'-CATCGTGGAGCAGAT-3' ; TMEM49-Exon12-Reverse- 5'-CAAGCGCTGCTGGATTC-3'.

Mouse models of cardiac remodeling. Eight-week-old mice underwent either a sham operation or were subjected to pressure overload induced by TAC as previously described (16). Cardiac hypertrophic agonist angiotensin II (2 mg/kg/d) (American Peptide) or saline were administered using osmotic minipumps (model 2002, Alzet) subcutaneously

implanted dorsally in 8-week-old male mice. Mice were sacrificed 14 days after AngII administration or 21 days following TAC.

miR-21$^{-/-}$ animals were bred to animals harboring the αMHC-Calcineurin transgene as previously described (17). Male animals heterozygous for miR-21 mutant allele and positive for the transgene were bred to miR-21 heterozygous transgene negative females. Offspring were sacrificed at 16 weeks of age.

To mimic MI adult C57Bl6 male mice were anesthetized with 2.4% isoflurane and placed in a supine position on a heating pad (37°C). Animals were intubated with a 19G stump needle and ventilated with room air, using a MiniVent mouse ventilator (Hugo Sachs Elektronik; stroke volume, 250 ul, respiratory rate, 210 breaths per minute). Via left thoracotomy between the fourth and fifth ribs, the LAD was visualized under a microscope and ligated by using a 6–0 prolene suture. Regional ischemia was confirmed by visual inspection under a dissecting microscope (Leica) by discoloration of the occluded distal myocardium. Sham operated animals underwent the same procedure without occlusion of the LCA. Mice were sacrificed 21 days following MI.

Histological analysis and Fibrosis Quantitation. Tissues used for histology were incubated in Krebs-Henselheit solution, fixed in 4% paraformaldehyde, sectioned, and processed for Masson's Trichrome staining. Fibrosis was quantitated using Adobe Photoshop. Fibrosis is represented as the area of trichrome positivity as a percentage of total left ventriclular area.

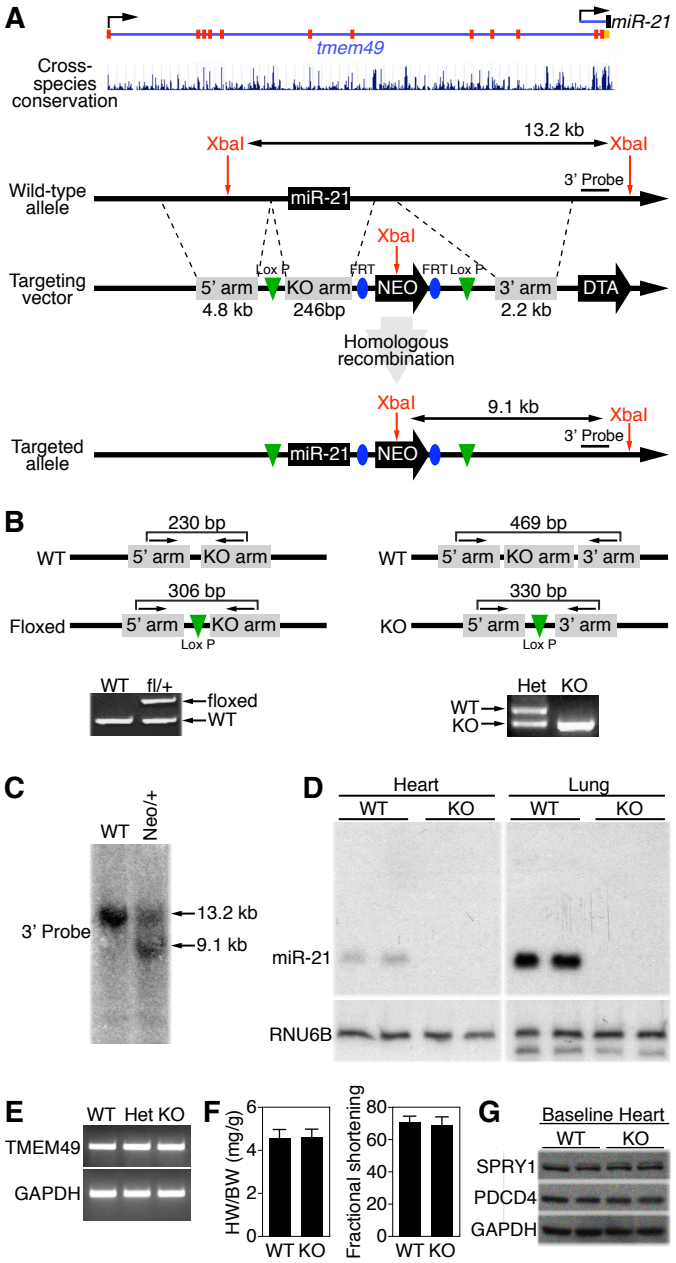
Western blotting. Total heart lysate was prepared by homogenizing tissue in RIPA lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1%

SDS, 1% sodium deoxycholate) and was resolved by SDS-PAGE, and analyzed to detect PDCD4, ERK1/2, phospho-ERK1/2, and GAPDH using 10 μg protein per sample. The proteins were transferred to PVDF membrane. Santa Cruz Western Blotting Luminol Reagent (Santa Cruz) was used for detection following the manufacturer's instructions. Primary antibodies used include rabbit polyclonal PDCD4 antibody (600-401-956, Rockland) at a dilution of 1:2000, rabbit polyclonal ERK1/2 (9102, Cell Signaling) at a dilution of 1:1000, rabbit polyclonal phospho-ERK1/2 (9101, Cell Signaling) at a dilution of 1:1000, rabbit polyclonal SPRY1 (ab21020, Abcam), and mouse monoclonal GAPDH (MAB374, Millipore) at a dilution of 1:10,000. HRP-conjugated secondary antibodies were used according to the manufacturer's instructions.

LNA-based knockdown of miR-21. The LNA-antimiR and LNA scramble control oligonucleotides were synthesized as unconjugated and fully phosphorothiolated oligonucleotides (Santaris Pharma, Denmark). The perfectly matching LNA-antimiR oligonucleotide was complementary to nucleotides 2–9 in the mature miR-21 sequence. A 8 nt LNA scramble sequence oligonucleotide was used as a control. For Cos cell transfection, cells were transfected with either a miR-21 expression vector or LNA-antimiR with Lipofectamie 2000 (Invitrogen) at a concentration of 10 nM. C57BL/6 mice were injected intravenously at the indicated timepoints with the indicated doses of antimiR-21, LNA control or a comparable volume of saline, after which the tissues were collected at the days indicated (Suppl. Fig. 6).

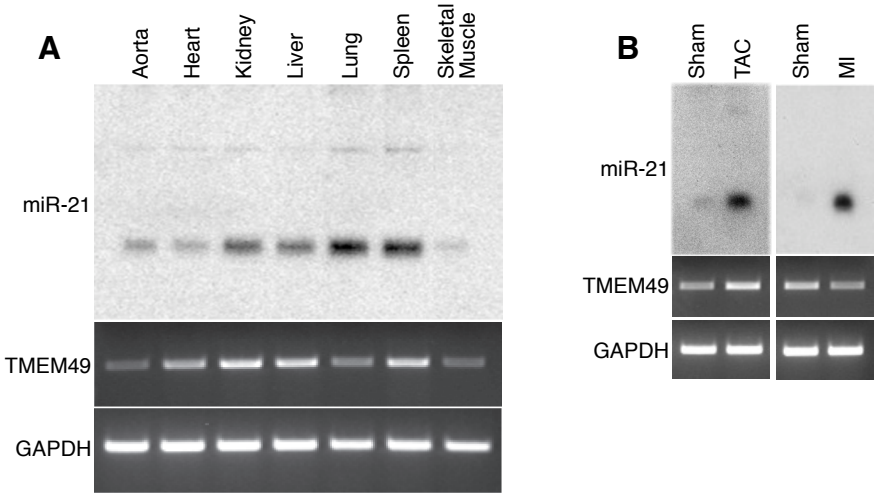
Transthoracic echocardiography. Evaluation of animals represented in Figure 3 was performed on mice sedated with 5% isoflurane. All other animals were not sedated for evaluation. Cardiac function and heart dimensions were evaluated by two-dimensional

echocardiography using a Visual Sonics Vevo 770 Ultrasound (Visual Sonics, Canada) and a 30-MHz linear array transducer. M-mode tracings were used to measure anterior and posterior wall thicknesses 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). The data were analyzed by a single observer blinded to mouse genotype. LV fractional shortening (FS) was calculated according to the following formula: FS (%) = [(LVIDd - LVIDs)/LVIDd] x 100.



Supplemental Figure 1. Generation of miR-21 mutant mice

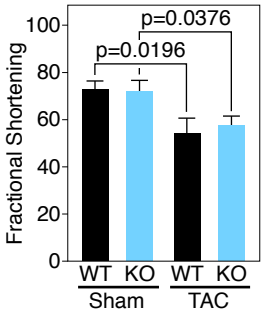
A. Schematic representation of the mouse miR-21 locus and targeting strategy. miR-21 is expressed as an overlapping transcript of TMEM49. The position of the 3' probe used for Southern blot is shown. Red boxes represent exons of the TMEM49 gene. DTA represents the selectable marker diptheria toxin A.
B. Genotyping PCR approach to confirm targeting and miR-21 deletion. The positions of the PCR primers used for genotyping mutant alleles are marked with arrows and the expected lengths for the PCR products are indicated.
C. Southern blot analysis of miR-21 mutant alleles. Genomic DNA was digested with XbaI and hybridized to a 3' probe. WT, wild-type allele; Neo/+, conditional allele.
D. Northern blot analysis for miR-21 in hearts and lungs of either wild-type (WT) or knockout (KO) mice indicating efficient deletion of miR-21. RNU6B is used as a loading control.
E. RT-PCR of TMEM49 in wild-type (WT), heterozygous mutant (het) and knockout (KO) animals indicates that miR-21 deletion does not interfere with the expression of TMEM49. GAPDH was used as a loading control.
F. Morphometric and functional analysis of hearts show that miR-21 deletion has no effect on heart size or function at 1 year of age, as indicated by the ratio between heart weight and body weight (HW/BW) and fractional shortening (FS) respectively. n=4 individuals per group.
G. Western blot analysis indicates comparable levels of SPRY1 and PDCD4 in WT and miR-21-/- (KO) hearts.



Supplemental Figure 2. Expression of miR-21 and TMEM49.

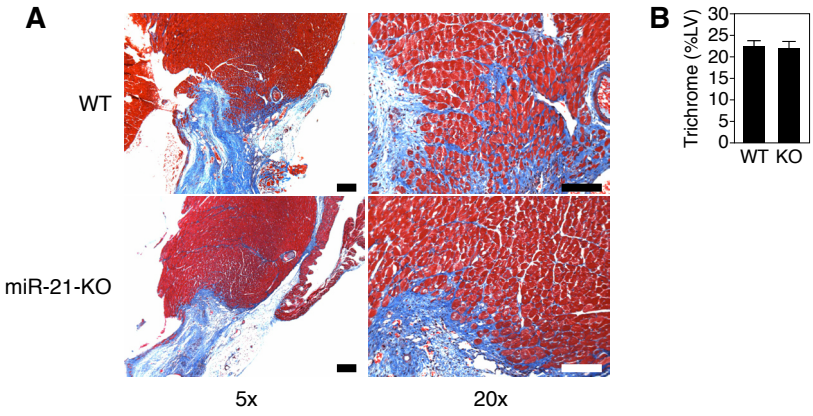
A. miR-21 was detected in the indicated mouse tissues by Northern blot. TMEM49 and GAPDH mRNAs were detected by RT-PCR.

B. miR-21 was detected in mouse heart following sham operation or TAC for 21 days. TMEM49 and GAPDH mRNAs were detected by real time PCR.



Supplemental Figure 3. Measurement of cardiac function by fractional shortening.

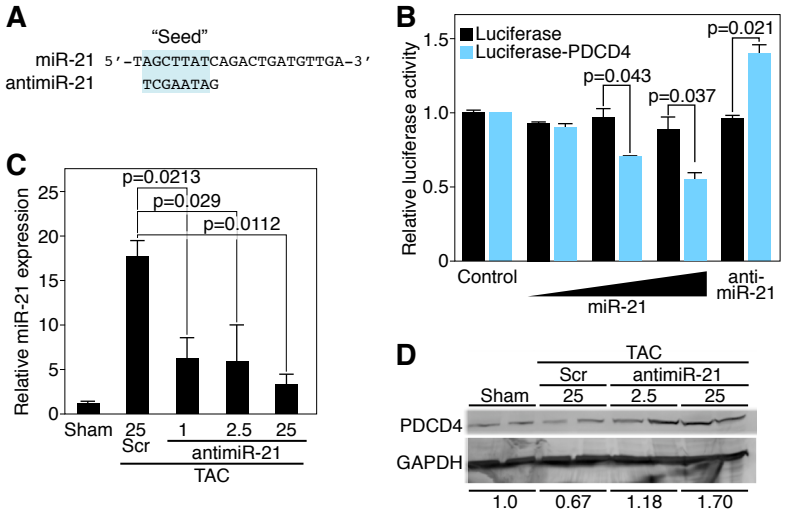
Fractional shortening was determined by echocardiography on wild type and miR-21 KO mice following sham operation or TAC for 21 days. Cardiac function is reduced comparably in mice of both genotypes following TAC. Data represent n = 8 for WT sham, n = 8 for KO sham, n = 11 for WT TAC, and n = 10 for KO TAC.



Supplemental Figure 4. Fibrosis and scar formation following myocardial infarction.

A. Wild type and miR-21 KO mice were subjected to LAD ligation to induce myocardial infarction (MI). Hearts were isolated 21 days later, sectioned and stained with Masson's trichrome. Bar = 100 um. Comparable fibrosis and scar formation are seen in mice of both genotypes.

B. Quantification of fibrosis in WT and KO hearts subjected to LAD. Fibrosis is displayed as the percentage of trichrome positive left ventriclular area. Data represent n=3 per condition.



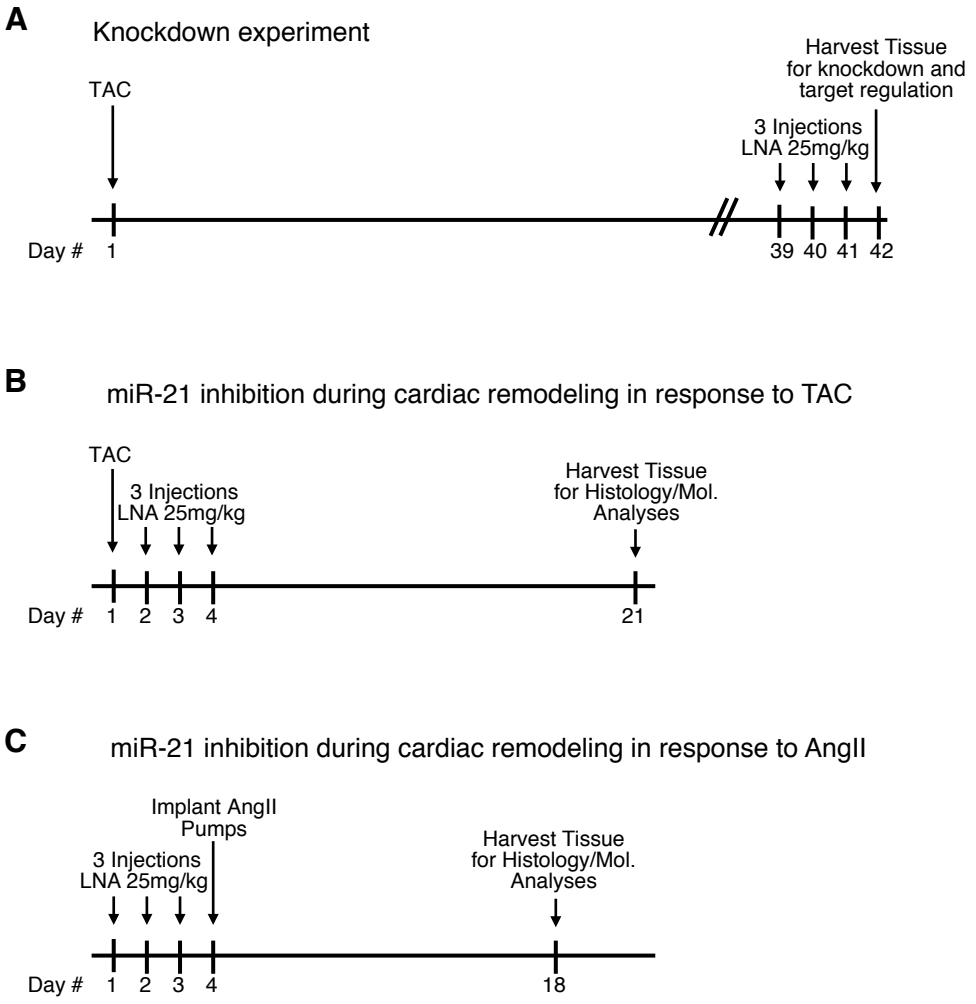
Supplemental Figure 5. Silencing of cardiac miR-21 using an LNA-antimiR

A. antimiR-21 is a fully LNA-modified phosphorothioate oligonucleotide complementary to the seed region of mature miR-21.

B. Relative activity of a luciferase reporter fused to the 3′ UTR of PDCD4 displays dose-dependent repression by CMV-driven miR-21, whereas treatment with antimiR-21 derepresses the luciferase reporter in COS-1 cells. Data represents the average of three experiments.

C. Real-time RT-PCR analysis of miR-21 was performed on cardiac tissue harvested from animals subjected to either sham or TAC surgeries. Animals subjected to TAC surgeries were injected with either 25 mg/kg LNA scrambled control (scr), 1 mg/kg antimiR-21 (1), 2.5 mg/kg antimiR-21 (2.5), or 25 mg/kg antimiR-21. TAC induces miR-21 expression in the LNA scrambled control treated animals, whereas treatment with antimiR-21 significantly inhibits miR-21 levels. Details of the injection scheme are provided in Supplemental Figure 4. Data shown represent n = 5 per condition.

D. Western blotting for PDCD4 on cardiac tissue after antimiR-21 treatment indicates a dose-dependent increase in PDCD4. GAPDH was used as a loading control.

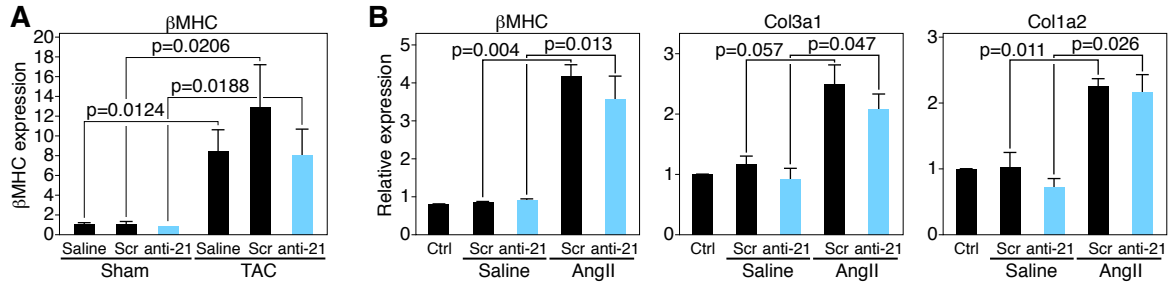


Supplemental Figure 6. Schematic diagrams of anti-miR-21 knockdown experiments.

A. TAC was performed to up-regulate miR-21 expression. After 39 days, mice were injected intravenously on three consecutive days with Tiny-21 (25 mg/kg) and hearts were isolated on day 42 and analyzed for miR-21 expression.

B. TAC was performed and mice were injected intravenously on three consecutive days with Tiny-21 (25 mg/kg) and hearts were isolated on day 21 and analyzed for histology, miR-21 expression and miR-21 target expression.

C. Mice were injected intravenously on three consecutive days with Tiny-21 (25 mg/kg) and on day 4 were implanted with AngII pumps to induce pathological cardiac remodeling. After 18 days, hearts were isolated and analyzed for histology and miR-21 expression.



Supplemental Figure 7. Cardiac stress response after antimiR-21 treatment

A. Real-time RT-PCR analysis of βMHC expression in cardiac tissue of animals treated as indicated. Data shown represent n=5 for sham conditions and n=10 for TAC conditions.

B. Real-time RT-PCR analysis of the cardiac stress induced genes βMHC, Col3a1, and Col1a2 after the indicated treatment compared to untreated animals. Data represent n=2 for Ctrl, and n=3 for all other conditions.