Therapeutic inhibition of the miR-34 family attenuates pathological cardiac remodeling and improves heart function

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MicroRNAs are dysregulated in a setting of heart disease and have emerged as promising therapeutic targets. MicroRNA-34 family members (miR-34a, -34b, and -34c) are up-regulated in the heart in response to stress. In this study, we assessed whether inhibition of the miR-34 family using an s.c.-delivered seed-targeting 8-mer locked nucleic acid (LNA)-modified antimiR (LNA-antimiR-34) can provide therapeutic benefit in mice with preexisting pathological cardiac remodeling and dysfunction due to myocardial infarction (MI) or pressure overload via transverse aortic constriction (TAC). An additional cohort of mice subjected to MI was given LNA-antimiR-34a (15-mer) to inhibit miR-34a alone as a comparison for LNA-antimiR-34. LNA-antimiR-34 (8-mer) efficiently silenced all three miR-34 family members in both cardiac stress models and attenuated cardiac remodeling and atrial enlargement. In contrast, inhibition of miR-34a alone with LNA-antimiR-34a (15-mer) provided no benefit in the MI model. In mice subjected to pressure overload, LNA-antimiR-34 improved systolic function and attenuated lung congestion, associated with reduced cardiac fibrosis, increased angiogenesis, increased Akt activity, decreased atrial natriuretic peptide gene expression, and maintenance of sarcoplasmic reticulum Ca\(^{2+}\) ATPase gene expression. Improved outcome in LNA-antimiR-34–treated MI and TAC mice was accompanied by up-regulation of several direct miR-34 targets, including vascular endothelial growth factors, vinculin, protein O-fucosyltransferase 1, Notch1, and semaphorin 4B. Our results provide evidence that silencing of the entire miR-34 family can protect the heart against pathological cardiac remodeling and improve function. Furthermore, these data underscore the utility of seed-targeting 8-mer LNA-antimiRs in the development of new therapeutic approaches for pharmacologic inhibition of disease-implicated miRNA seed families.

The prevalence of symptomatic heart failure is increasing owing to the growing elderly population (1). Existing therapies typically slow, rather than prevent or reverse, the progression of heart failure. Furthermore, therapies that are effective often have serious side effects (2). Thus, there is an urgent need for well-tolerated therapies with the ability to significantly attenuate adverse cardiac remodeling and improve function of the failing heart. With rapid advances in understanding of the regulation and roles of small, noncoding RNAs known as microRNAs (miRNAs) in cardiac pathology, and developments in antisense oligonucleotide chemistries, the therapeutic potential of inhibition of miRNAs in cardiac disease settings is considered high (3, 4). miRNAs represent attractive therapeutic targets because they are small (~22 nucleotides) and can be efficiently inhibited in vivo (3, 5). The translation of a miRNA-based therapy from mice (6) to primate (7) and into clinical trials has already been demonstrated with miravirsen, an inhibitor of miR-122 for the treatment of hepatitis C virus infection. Results from a Phase 2a trial indicated that the treatment was well tolerated in patients infected with hepatitis C virus and was associated with continuous and prolonged antiviral activity beyond the period of treatment (8). This finding has sparked enthusiasm and anticipation for the development of other miRNA-based drugs.

Phosphoinositide 3-kinase [PI3K(p110\(\alpha\))] lies downstream of the insulin-like growth factor 1 receptor and is an essential mediator of physiological heart growth, that is, normal postnatal heart growth or heart growth in response to chronic exercise training (9–11). PI3K(p110\(\alpha\)) also mediates the cardioprotective properties of exercise (12), and elevated PI3K(p110\(\alpha\)) activity protects the heart against insults, including pressure overload, dilated cardiomyopathy, and myocardial infarction (MI) (9, 13–15). We previously reported that miR-34a expression was increased in the mouse heart in a setting of stress (i.e., MI) and suppressed in a setting of protection, owing to transgenic expression of PI3K(p110\(\alpha\)) (13). We also observed elevated expression of other members of the miR-34 family (i.e., miR-34b and miR-34c) in mouse hearts after MI (13). Furthermore, expression of miR-34a and/or miR-34b and c was found to be elevated in cardiac tissue from patients with heart disease (16, 17). Thus, we hypothesized that inhibition of the entire miR-34 family in mice with preexisting cardiac dysfunction would be beneficial and demonstrate greater therapeutic potential compared with inhibition of miR-34a alone.

Several previous studies have examined inhibition of individual miRNAs in the heart using chemically modified antisense oligonucleotides known as antimiRs (15-22 mer) (3, 4). Modifications used to enhance the binding affinity of antimiRs to their cognate miRNAs and to facilitate cellular uptake for in vivo delivery include incorporation of 2′-O-methyl, 2′-O-methoxyethyl, locked nucleic acid (LNA), 3′-chitosan conjugation (antagomiRs), and phosphorothioate backbone modification (3, 5, 7, 18, 19). More recently, we described an approach to inhibit entire miRNA families using seed-targeting 8-mer LNA-antimiR oligonucleotides known as LNA-antimiRs (20). Key advantages of this methodology include the ability to simultaneously knock down entire miRNA families, easy formulation in saline for delivery via multiple routes, negligible off-target effects, and no evidence of toxicity. Collectively, these features make tiny LNAs potentially attractive for development of therapeutic strategies for inhibiting disease-associated miRNA families. We report here that s.c. delivery of an 8-mer LNA-modified antimiR-34 efficiently inhibits the miR-34 family (miR-34a, b, and c), attenuates MI-induced remodeling and dysfunction, and improves cardiac function in a model of pressure overload-induced pathological hypertrophy and dysfunction.


Conflict of interest statement: S.O. and S.K. are employees of Santaris Pharma, a clinical-stage biopharmaceutical company that develops RNA-targeted therapeutics. This article is a PNAS Direct Submission.

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Results
The main focus of this study was to inhibit the entire miR-34 family in settings of cardiac stress using an 8-mer LNA oligonucleotide complementary to the seed region of the miR-34 family (20). A small cohort of mice was also given a 15-mer LNA-modified antimiR-34a specifically targeting miR-34a, for comparison with the entire LNA. We previously reported that miR-34a increased in hearts of MI mice compared with sham mice, and that expression was inversely correlated with cardiac function, suggesting that inhibition of miR-34a might be beneficial (13). The microarray dataset from which miR-34a was identified (13) also showed elevated miR-34b in hearts of MI mice compared with sham mice, along with a trend toward an increase in miR-34c, which we validated by quantitative RT-PCR (RT-qPCR) (SI Appendix, Fig. S1A and B). Thus, we hypothesized that inhibition of the entire miR-34 family would represent a more effective treatment strategy than inhibition of miR-34a alone.

Inhibition of the miR-34 Family, but Not of miR-34a Alone, Attenuates MI-Induced Morphological Changes. In adult control mice, we first demonstrated that administration of a single dose of the 15-mer LNA-antimiR-34a on 3 consecutive days inhibited miR-34a in the heart as early as 1 d after administration, and that miR-34a inhibition persisted for 2 mo after the last dose, compared with hearts from LNA-control treated mice (SI Appendix, Fig. S2 A and B). We next assessed the impact of inhibiting miR-34a alone or the entire miR-34 family in mice with preexisting cardiac dysfunction due to MI. Mice subjected to MI (i.e., permanent occlusion of the left anterior descending coronary artery) or the sham operation were dosed with LNA control (15-mer or 8-mer), LNA-antimiR-34 (8-mer), or LNA-antimiR-34a (15-mer) at 2 d after surgery (SI Appendix, Fig. S3A). Based on morphological and functional parameters (8 wk post-MI), no differences between the 15-mer and 8-mer LNA control-dosed mice were seen; thus, these groups were combined (SI Appendix, Fig. S4). The 15-mer LNA-antimiR-34a and the 8-mer LNA-antimiR-34 antagonized miR-34a in the hearts of MI mice compared with LNA control-dosed mice (Fig. 1 A and B). The 8-mer LNA-antimiR-34 also inhibited miR-34b and miR-34c (Fig. 1B), miR-34b and miR-34c were not significantly inhibited in the 15-mer LNA-antimiR-34a-dosed mice (SI Appendix, Fig. S5A). However, the high homology among the miR-34 family members could allow cross-hybridization of the 15-mer LNA-antimiR-34a with miR-34b and miR-34c, which likely explains the weak inhibition observed for miR-34b and miR-34c (~60% of that of LNA control; SI Appendix, Fig. S5A and B).

Mean infarct size assessed at dissection was similar in LNA control-treated, LNA-antimiR-34–treated, and LNA-antimiR-34a–treated MI mice (28.7%, 26.1%, and 28.9%, respectively) (SI Appendix, Table S1). At 8 wk post-MI, LNA-control treated MI mice displayed significant pathology, including increased heart weight (HW)/tibia length (TL) ratio (Fig. 1C), a trend toward an increased lung weight (LW)/TL ratio (Fig. 1D; p = 0.09), and an increased atrial weight (AW)/TL ratio (Fig. 1E and F) compared with sham mice. These morphological changes were blunted by the 8-mer LNA-antimiR-34 (Fig. 1 C–E), but not with the 15-mer LNA-antimiR-34a (SI Appendix, Table S1). Of note, neither LNA-antimiR-34 nor LNA-antimiR-34a had an effect on body weight, spleen weight, or kidney weight compared with LNA control-dosed sham or MI mice (SI Appendix, Table S1).

Inhibition of miR-34 Attenuates Pathological Left Ventricular Remodeling After MI. At 2 d post-MI, but before LNA administration, mice displayed significant left ventricular (LV) remodeling, including increased LV dimensions and reduced fractional shortening, compared with sham mice (Fig. 1G and SI Appendix, Table S2). At 8 wk post-MI, fractional shortening decreased further in the LNA-control treated MI mice (Fig. 1G), and thinning of the LV walls was seen (Fig. 1H and SI Appendix, Table S2). Fractional shortening was higher and LV walls were thicker in LNA-antimiR-34–treated MI mice compared with LNA control-dosed MI mice at 8 wk post-MI (Fig. 1 G and H and SI Appendix, Table S2). Of note, fractional shortening was not improved in LNA-antimiR-34a–dosed MI mice (SI Appendix, Fig. S6).

MI is accompanied by an inflammatory response and collagen deposition. Improved outcome in LNA-antimiR-34–treated MI mice was associated with reduced transcription of the inflammatory marker IL-6 in the infarct zone, along with a similar trend in the remote zone (SI Appendix, Fig. S7A). Collagen Iα1 (Col1α1) gene expression was elevated in the infarct zone of all MI groups compared with sham mice and did not differ among groups, although there was a trend toward lower Col1α1 expression in the remote zone of LNA-antimiR-34–treated MI mice compared with LNA control-treated MI mice (Fig. S7B). The α-myosin heavy chain (MHC)/β-MHC ratio (a molecular marker of contractility) was diminished or tended to be reduced in LNA control and LNA-antimiR-34–treated MI mice, but was not significantly suppressed in LNA-antimiR-34–treated MI mice (SI Appendix, Fig. S7C).

We next assessed the expression of several validated miR-34a targets that have been implicated in improved outcomes in models of cardiac ischemia and/or cell survival, including cyclin
Notch1 (critical for cardiac repair after MI) (25), and protein phenotype identified the miR-34 family, which also may contribute to the favorable expression of vinculin (a similar trend was apparent for a relative to snoU6 by RT-qPCR. In contrast, was increased in LNA-antimiR-34-treated MI mice, but not in LNA-antimiR-34-treated MI mice (SI Appendix, Fig. S7D). In contrast, was increased in LNA-antimiR-34–treated MI mice, but not in LNA-antimiR-34–treated MI mice (SI Appendix, Fig. S7E), and a similar trend was apparent for Ppp1r10 (SI Appendix, Fig. S7F).

Finally, we measured the expression of predicted targets of the miR-34 family, which also may contribute to the favorable phenotype identified in LNA-antimiR-34–treated MI mice. Expression of vinculin (Vcl, essential for cardiomyocyte stability) (24), Notch1 (critical for cardiac repair after MI) (25), and protein O-fucosyltransferase 1 (Pofut1, required for functional Notch signaling in vivo) (26) were elevated in the remote zone of LNA-antimiR-34–treated MI mice compared with LNA control-treated MI mice (Fig. 2 A–C). Bcl6 also tended to be elevated (Fig. 2D), which protects cardiomyocytes from inflammation (27). We also evaluated semaphorin 4B (Sema4b) expression, because in addition to being a miR-34 family target, Sema4b is associated with PI3K-mediated cardiac protection in an MI setting (13). Sema4b expression was increased in LNA-antimiR-34–treated MI mice compared with LNA control-treated MI mice (Fig. 2E, Left) and was positively correlated with fractional shortening (Fig. 2E, Right).

Inhibition of miR-34 Prevents Pressure Overload-Induced LV Remodeling and Improves Cardiac Function. To determine whether inhibition of miR-34 could improve systolic function in a mouse model with preexisting pathological hypertrophy and systolic dysfunction, we subjected mice to pressure overload via transverse aortic constriction (TAC) for 5 wk, followed by 6 wk of treatment with LNA-antimiR-34 (SI Appendix, Fig. S3B). Before treatment, LV remodeling in response to TAC for 5 wk was confirmed by echocardiography. TAC mice displayed increased LV wall thickness and depressed fractional shortening compared with presurgery values and sham mice (SI Appendix, Table S3). Mice were then randomly assigned to receive LNA-antimiR-34 or LNA control for 6 wk.

LNA treatment (LNA control or LNA-antimiR-34) had no impact on body weight during the study period (SI Appendix, Table S3). Consistent with our previous observation of increased expression of the miR-34 family in LV of the MI model (SI Appendix, Fig. S1) (13), TAC also induced increased expression of miR-34a, b, and c in the heart compared with sham mice (Fig. 3A; LNA control TAC vs. LNA control sham). The miR-34 family was antagonized in the hearts of both sham and TAC mice given 8-mer LNA-antimiR-34, as assessed by RT-qPCR (Fig. 3D). We also
assessed inhibition of miR-34a by Northern blot analysis (Fig. 3A). Detection of a shifted miR-34a:LNA-antimiR-34 band on Northern blots implies that LNA-antimiR-34 sequesters miR-34a in a stable heteroduplex, thereby inhibiting its function.

LNA control-treated TAC mice developed hypertrophy (increased HW:TL; Fig. 3 B and C), atrial enlargement (increased AW:TL; Fig. 3 B and D), and lung congestion (Fig. 3E). In contrast, each of these parameters was blunted and/or not significantly elevated (vs. sham) in LNA-antimiR-34–treated TAC mice (Fig. 3 C–E). LNA-antimiR-34 had no effect on kidney weight (SI Appendix, Table S4). The increased heart size in LNA control-treated TAC mice was associated with increased cardiomyocyte size and fibrosis (Fig. 3 F and G), which were attenuated in LNA-antimiR-34–treated mice.

Fractional shortening decreased by ~30% after 5 wk of pressure overload, associated with an increase in LV end-systolic dimension (Fig. 3 H and SI Appendix, Table S3). At 6 wk after LNA administration, there was no difference in fractional shortening in LNA control-treated TAC mice compared with pretreatment values at 5 wk after TAC (Fig. 3H). In contrast, LNA-antimiR-34 treatment was associated with increased fractional shortening in TAC-treated mice that was significantly improved compared with the same mice at 5 wk after TAC and in LNA-control treated TAC mice at 11 wk after TAC (Fig. 3H and SI Appendix, Fig. S8).

Inhibition of miR-34 in TAC Mice Is Associated with an Improved Cardiac Molecular Signature, Up-Regulation of miR-34 Targets, and Increased Angiogenesis and Vinculin. Cardiac dysfunction in LNA control-treated TAC mice was accompanied by increased expression of atrial natriuretic peptide (Anp; Fig. 4A) and βMHC (Fig. 4B), and decreased sarcoplasmic reticulum Ca2+ ATPase (Serca2a; Fig. 4A). Improved cardiac function in LNA-antimiR-34 treated TAC mice was associated with reduced Anp expression, more favorable Serca2a expression (Fig. 4A), and a higher αMHC/βMHC ratio (Fig. 4B). The phosphorylation of Akt was also elevated in hearts from LNA-antimiR-34 treated TAC mice (Fig. 4C).

To investigate the mechanisms by which LNA-antimiR-34 treatment might improve cardiac function in a setting of TAC, we measured the expression of several miR-34 family targets, including VEGFs, POFUT1, and VCL. VEGF-A and VEGF-B have cardioprotective properties (28, 29) and are experimentally validated targets of miR-34 family members (30, 31). In the present study, protein expression of VEGF-A was increased in the hearts of LNA-antimiR-34–treated TAC mice compared with LNA control-treated TAC mice, and a trend toward increased VEGF-B protein expression accompanied by significantly increased Vegfb transcription was also seen (Fig. 4D). Increased VEGF expression in the hearts of LNA-antimiR-34–treated TAC mice was associated with increased capillary density (Fig. 4E).

There was a trend toward increased POFUT1 protein expression in LNA-antimiR-34–treated TAC mice that was associated with increased gene transcription (SI Appendix, Fig. S9 A and B). As seen in the MI model, vinculin was elevated in hearts from TAC mice treated with LNA-antimiR-34 (Fig. 4F). The intensity of vinculin staining appeared to be reduced at the intercalated disks in myocytes from LNA control-treated TAC mice, but not in LNA-antimiR-34–treated mice, compared with sham mice (Fig. 4F).

Vinculin, Sema4b, Pofut1, and Bcl6 Are Targets of the miR-34 Family. Vinculin, Sema4b, Pofut1, and Bcl6 were predicted conserved targets of the miR-34 family in mouse, rat, human, and chimpanzee using TargetScan 6.2 (Fig. 5A and SI Appendix, Fig. S10). To assess whether miR-34a, b, and c directly bind the 3′ UTR of each predicted target mRNA, we performed luciferase reporter assays in HEK 293T cells and the cardiomyoblast H9c2 cell line (Fig. 5B and SI Appendix, Fig. S10). Overexpression of miR-34a, b, and c inhibited luciferase activity of reporter constructs containing the 3′ UTR segment of vinculin, Sema4b, Pofut1, or Bcl6. Importantly, luciferase activity was not affected by noncoding RNAs that are not predicted to target the 3′ UTRs of these genes (miR-Ctrl; Fig. 5B and SI Appendix, Fig. S10), demonstrating specificity of the miR-34 family in targeting the seed region of each 3′ UTR. These data, together with the up-regulation of these targets in hearts from LNA-antimiR-34–treated TAC or MI mice, suggest that vinculin, Sema4b, Pofut1, and Bcl6 are bona fide targets of miR-34a, b, and c.

**Fig. 4.** LNA-antimiR-34 treatment is associated with a more favorable cardiac molecular profile in TAC mice, regulates miR-34 targets, and increases angiogenesis. (A and B) Northern blots and quantification of Anp and Serca2a relative to Gapdh and αMHC relative to βMHC in sham and TAC mice dosed with LNA control (CON; c) or LNA-antimiR-34 (a). βMHC was not detected in sham mice. n = 3–4 for sham mice; n = 4–8 for TAC mice. *P < 0.05 vs. both sham groups; †P < 0.05 vs. TAC LNA control; ‡P < 0.05 vs. sham LNA control (unpaired t test). (C and D) Western blots and quantification of p-Akt relative to TAKT and VEGF-A, VEGF-B, and VCL relative to GAPDH in hearts from TAC mice dosed with LNA control (c) or LNA-antimiR-34 (a). RT-qPCR analysis of Vegfb standardized to Hprt1. n = 3–4 for LNA control TAC mice; n = 4–5 for LNA-antimiR-34 TAC mice. *P < 0.05 vs. TAC LNA control. (E) Left: Capillary density in ventricular sections from TAC mice. Wheat germ agglutinin (WGA) stains myocyte membranes (green), and isocitron B4 stains capillaries (red). (Scale bar: 20 μm.) (Right) Quantification (n = 3/group). *P < 0.05 vs. TAC LNA control. (F) Representative images of vinculin staining in hearts from sham and TAC. White arrows highlight intercalated disks. n = 3–4 hearts stained/group. (Scale bar: 20 μm.)
The present study has produced four major findings. First, the miR-34 family was efficiently and chronically silenced using an s.c.-delivered seed-targeting 8-mer LNA. Second, inhibition of the miR-34 family, but not of miR-34a alone, inhibited MI-induced LV remodeling and atrial enlargement, highlighting the therapeutic potential of targeting an entire family. Third, inhibition of the miR-34 family improved systolic function in mice with pre-existing pathological hypertrophy and dysfunction due to chronic pressure overload. Fourth, we experimentally validated four critical miR-34 family targets, including VEGFs (30, 31), and targeting the entire miR-34 family would provide a better therapeutic approach.

Improved systolic function in LNA-antimiR-34–treated mice was associated with reduced fibrosis, increased capillary density, increased cardiac Akt activation, lower Anp expression, elevated dMHC/βMHC ratio, and better preserved Serca2a gene expression. Fig. 6 illustrates possible mechanisms by which inhibition of the miR-34 family could provide cardiac protection in the TAC and MI models based on regulation of previously validated miR-34 family targets, including VEGFs (30, 31), and targets that we have experimentally validated in the present study (vinculin, Pofut1, Sema4b, and Bcl6). VEGF-A is critical to increasing capillary density, and the reduced coronary angiogenesis in cardiac stress models contributes to the transition to heart failure (29). Vinculin is necessary for the preservation of cardiac contractile and electrical function, and it regulates the expression of 8-mer LNA-antimiR can inhibit the entire miR-34 family and provide a benefit in two severe models of cardiac stress with pre-existing systolic dysfunction.

The number of new cardiovascular drugs entering the market has been low, in part because many agents under development have not demonstrated clear patient benefits in efficacy over the current standard of care (3). The limited translation of promising approaches identified in basic research laboratories may be due, at least in part, to experimental designs that manipulate genes or deliver therapeutic agents just before or simultaneously with cardiac insults, as opposed to settings of established pathology. Because patients typically seek medical advice once symptoms are present or after an event such as a heart attack, we designed experiments to assess the potential benefit of inhibiting the miR-34 family in two cardiac stress models with significant preexisting LV remodeling and cardiac dysfunction. In the MI study, LNA-antimiR-34 was unable to completely prevent LV remodeling over the 8-wk study period post-MI. However, LNA-antimiR-34–treated MI mice had smaller LV dimensions, increased LV wall thicknesses, and better systolic function compared with LNA control-treated MI mice. Furthermore, MI mice given LNA-antimiR-34 had lower heart, lung, and atrial weights compared with those given LNA control. Of note, the 15-mer LNA-antimiR-34a provided no significant benefit in the MI setting. Our previous work and present results demonstrate increased miR-34b and miR-34c expression in the heart in MI and pressure overload settings. This likely explains why pharmacologic inhibition of the miR-34 family was more effective than inhibition of miR-34a alone. Of note, Dimmeler et al. (23) reported that inhibition of miR-34a was effective in improving ejection fraction in an acute MI setting. Differences between the current study and Dimmeler et al. (23) include the model of MI (acute vs. chronic), time of administration, and type of antimiR delivered (3′-cholesterol-conjugated 2′ O-methyl-modified antimiR vs. unconjugated LNA-modified antimiR with a complete phosphorothioate backbone). Whether LNA-antimiR-34a would provide a benefit in the less severe model of acute MI remains to be elucidated. However, given that expression of the entire miR-34 family was elevated in an MI setting, we hypothesize that inhibition of the entire miR-34 family would provide a better therapeutic approach.
of proteins residing at the intercalated disk (24). Pofut1 is essential for functional Notch signaling, and heart-specific Pofut1 KO embryonic heart defect carriers display cardiac defects (26). Notch 1 (a targeted target of miR-34a) has been implicated as an important mediator of cardiac repair after MI (25). Bcl6 is known to protect mature cardiomyocytes from inflammation (27), and Sema4b has been shown to inhibit IL-6 production by basophils (36). Sema4b was also positively correlated with systolic function in an MI setting (Fig. 2E). miRNAs have many target genes, and the present study does not delineate the contribution of identified up-regulated targets in LNA-antimiR-34-treated mice mediating protection. Future in vivo studies incorporating KO models of these targets are needed to comprehensively address this question. However, the dysregulation of any of these targets has been shown to mediate cardioprotection, we believe that up-regulation of these targets is likely to contribute to the protection observed.

Of note, miR-34 family members also have been recognized as tumor suppressor miRNAs. Given that the miR-34 family has been implicated in the p53 tumor suppressor network, and that p53 pathway defects are common features of human cancer (37), miR-34 replacement therapy is considered a promising therapeutic approach (38). Recent reports demonstrate that inhibition of the miR-34 family does not promote tumorigenesis, supporting the potential for therapeutic suppression of this family as a treatment for heart failure (39). However, because other studies have shown that the miR-34 family may be sufficient to modulate tumor progression (40), future therapeutic approaches might benefit from a targeted approach that can restrict inhibition to the heart. On the other hand, miR-34 replacement in patients with cancer requires caution, because it may make the heart susceptible to dysfunction in a setting of stress.

In summary, our results provide evidence that inhibition of the miR-34 family can improve cardiac function in mice with preexisting pressure overload-induced hypertrophy and systolic dysfunction, and can attenuate pathological remodeling after MI. Furthermore, our data highlight the utility of seed-targeting 8-mer LNA-antimiRs for the pharmacologic inhibition of disease-implied miRNA seed families. Given that LNA-based therapies have already entered clinical trials, this approach has the potential for translation.

**Materials and Methods**

**Animals.** Animal care and experimental procedures were approved by the Alfred Medical Research and Education Precinct’s Animal Ethics Committee.

**Administration of LNA-AntimiR Oligonucleotides to Control Mice and Cardiac Stress Models.** Details of synthesis of LNA-antimiR-34a (15-mer), LNA-antimiR-34 (8-mer), and LNA-control sequences, as well as delivery of LNA-antimiR oligonucleotides in control adult mice and cardiac stress models (MI and TAC), are provided in SI Appendix. Details of luciferase assays and histological, molecular, and statistical analyses are also provided in SI Appendix.

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Bernardo et al.
Supporting Information (SI) Appendix (Methods, Legends, Figures and Tables)

Supporting Materials and Methods

Experimental Protocols

*Myocardial infarction (MI)*

Adult (10 weeks old) male C57BL/6 mice were subjected to either a sham or MI surgery as described (1). MI surgery is performed to simulate a ‘heart attack’ in patients. Cardiac function was assessed by echocardiography before surgery and 2 days post-surgery to confirm LV dilatation and systolic dysfunction as described (2). Mice were then randomly administered LNA-control, LNA-antimiR-34a or LNA-antimiR-34 subcutaneously (dosing regimen described below). Cardiac function was assessed 8 weeks post treatment, followed by tissue collection. Infarct size was calculated as a percentage of the infarct area over the entire LV area, as described (1).

*Pressure overload*

Adult (12 weeks old) male FVB/N mice were subjected to either a sham or pressure overload surgery (TAC) as reported (3). The TAC model is associated with pathological hypertrophy and systolic dysfunction within 4 weeks of surgery (3). This model simulates, to a degree, patients with chronic high blood pressure. Cardiac function was assessed before surgery and 5 weeks post-surgery. Mice were then administered either LNA-control or LNA-antimiR-34 subcutaneously for 6 weeks (details of dosing regimen described below). Cardiac function was assessed 6 weeks post treatment (11 weeks post-surgery), followed by tissue collection.

*LV structure and function*

Echocardiography (two-dimensional M-mode) was performed in anesthetized mice (1.8% isoflurane) utilizing a Philips iE33 ultrasound machine with a 15MHz linear array transducer, as described (4). LV wall thicknesses (LV posterior wall, LVPW; interventricular septum, IVS), LV
chamber dimensions (LV end-diastolic dimension, LVEDD; LV end-systolic dimension, LVESD), heart rate (HR), and fractional shortening (FS) were measured.

**LNA-antimiR synthesis and administration**

*Synthesis of LNA-antimiR-34a and LNA-antimiR-34*: The LNA-modified antimiR oligonucleotides were synthesized with a complete phosphorothioate backbone (5). The sequence of the 15-mer LNA-antimiR-34a was: 5´- AgCtaAGacAcTgCC – 3´ (LNA uppercase, DNA lowercase), and it was specifically designed to target miR-34a. The LNA-antimiR-34 was 8-mer in length, complementary to the seed region (5’- ACACTGCC – 3´), allowing for silencing of the entire miR-34-family (i.e. miR-34a, miR-34b and miR-34c)(5). The LNA-controls were synthesized with the following sequences: 5´- TcAtaCTatAtGaCA – 3´ and 5´- TCATACTA – 3´ (LNA uppercase, DNA lowercase). The LNA-control sequence had no perfect match binding sites in the transcriptome based on numerous databases, and has been validated with *in vitro* and *in vivo* assays and not shown to differ from untransfected/mock or saline samples (5).

*Delivery of LNA-antimiR oligonucleotides in control adult mice*: To confirm prolonged inhibition of miR-34a beyond the period of treatment with LNA-antimiRs, 10 week old adult male C57BL/6 mice were administered a single daily s.c. injection of LNA-control or LNA-antimiR-34a (25mg/kg/day) for 3 days. Hearts were collected from mice 1 day, 1 month and 2 months after the final dose to confirm effective inhibition of miR-34a in the heart.

*Delivery of LNA-antimiR oligonucleotides in the MI model*: Two days post surgery (after echocardiography), MI mice were dosed with LNA-control, LNA-antimiR-34a or LNA-antimiR-34 (25mg/kg/day s.c.) for 3 days (SI Appendix Fig 3A).
Delivery of LNA-antimiR oligonucleotides in the TAC model: The TAC model is less susceptible to cardiac complications with handling than the MI model, and at the time of the study it was unclear the degree to which pressure overload would increase the expression of miR-34a,b,c in the heart (treatment was not initiated until 5 weeks post-TAC). Thus, to ensure sufficient and continuous pharmacological inhibition in the TAC model, which is associated with pre-existing pathological hypertrophy and systolic dysfunction, mice were administered with a loading dose (25mg/kg s.c.) of LNA-control or LNA-antimiR-34 (Monday), 2 subsequent doses at 10mg/kg (Wednesday & Friday), followed by treatment three times per week (10mg/kg s.c.; Monday, Wednesday, Friday) for an additional 5 weeks i.e. total treatment period of 6 weeks (SI Appendix Fig 3B).

Impact of LNA-antimiR-34 on bone morphology: 10-12 week old adult male FVB/N mice were administered LNA-control or LNA-antimiR-34 as shown in SI Appendix Fig 3A. Tibia/femur were collected 2 weeks after the final dose to assess inhibition of miR-34a,b,c in bone.

RT-qPCR and Northern blotting

RNA: Total RNA was isolated from mouse heart, kidney and liver using TRI Reagent (Sigma-Aldrich, St Louis, MO) using the homogeniser PRO 200® (Harvard Apparatus, 72-1297) with a saw-tooth probe. For extraction of RNA from bone, tibia/femur was pulverized into powder using Mikro-Dismembrator S (Sartorius, 16s, 3000 speed) prior to homogenization in TRI Reagent.

RT-qPCR: For RT-qPCR analysis of miRNAs, 2μg of total RNA was DNASE treated with Ambion’s TURBO DNA-free kit (Life Technologies, Grand Island, NY) according to the manufacturer’s recommendations. To detect the level of miR-34a, miR-34b and miR-34c, RT-qPCR was performed using Taqman MicroRNA Assays (Life Technologies) according to the manufacturer’s instructions, using 10ng total RNA on an Applied Biosystems 7500 real-time PCR
instrument (Life Technologies). Expression was normalized against snoU6 using the $2^{-\Delta\Delta Ct}$ method of quantification.

For expression analysis of mRNA targets, fibrosis and inflammatory genes, cDNA was obtained by reverse transcription using oligo(dT) with the Superscript III kit (Life Technologies) according to manufacturer’s recommendations. RT-qPCR was performed using TaqMan probes (Life Technologies). Expression was normalized against Hypoxanthine phosphoribosyltransferase 1 (Hprt1) using the $2^{-\Delta\Delta Ct}$ method of quantification.

**miR Northern blotting:** Total RNA (15μg per sample) from heart tissue was electrophoresed in a 20% TBE acrylamide gel (Life Technologies) using High-density TBE sample buffer (Life Technologies) preheated at 70°C for 5 mins. Following electrophoresis, total RNA was transferred to Amersham Hybond™-N+ nylon membrane (GE Healthcare, Waukesha, WI) by electrophoresis. After transfer, the membrane was cross-linked in a UV-crosslinker and pre-hybridization carried out at 45°C for 30 mins in ULTRAhyb®-Oligo Hybridization Buffer (Life Technologies). Following pre-hybridization, the membrane was probed with LNA-modified oligonucleotides (6) (Exiqon, Denmark), complementary to mature microRNA-34a and sno-U6 (used as a loading control). 10 pmol of each LNA oligonucleotide was end-labelled with [$\gamma$-32P]ATP (Perkin Elmer, Waltham, MA) by using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) for 1 hour at 37°C. Hybridization was performed at 45°C overnight in ULTRAhyb®-Oligo Hybridization Buffer (Life Technologies). The labelled probes were heated for 5 mins at 100°C before addition to the hybridization buffer. After hybridization, the membranes were washed for 30 mins in NorthernMax® Low Stringency Wash Buffer (Life Technologies) at 45°C, followed by a high stringency wash in 0.5X SSC for 30 mins at 45°C (for LNA-oligonucleotide miR-34a) or 0.1X SSC for 30 mins at 45°C (for LNA-oligonucleotide sno-U6). The membranes were exposed using autoradiography film.
**mRNA Northern blotting:** Northern blotting was performed as previously reported except that 10 μg total RNA was used (7). Probes for Anp, Serca2a, αMHC, βMHC and Gapdh were generated as described (7-9).

**Western blotting**

Heart lysates were prepared as described (10). Western membranes were probed with p-(Ser473) AKT (Cell Signaling, #9271, 1:500), stripped and re-probed with total AKT (Cell Signaling, #9272, 1:2500), and VEGF-A (Santa Cruz, sc-152, 1:500), VEGF-B (Santa Cruz, sc-13083, 1:1000), POFUT1 (Novas Biologicals, NBP1-76940, 1:1000) or VCL (Sigma Aldrich, clone hVIN-1, V9131, 1:2000), stripped and re-probed with GAPDH (Santa Cruz, sc-32233,1:5000).

**Histological analyses and immunofluorescence**

Tissue samples (heart, liver and kidney) were fixed in 4% paraformaldehyde (PFA), paraffin embedded, sectioned (6μm), and stained with haematoxylin and eosin (H&E) to assess tissue morphology. For bone, tibia/femur was dissected, fixed in 4% PFA overnight, decalcified in 7% EDTA/PBS for 2 weeks at 4°C prior to paraffin embedding. Sections (8μm) were stained with 0.4% toluidine blue and 0.02% fast green (11, 12).

Cell area (wheat germ agglutinin stain, WGA), fibrosis (Masson’s trichrome), and angiogenesis (co-stained with isolectin B4 and WGA) were assessed as described (13, 14).

**Immunomicroscopy of vinculin:** Immunofluorescence was performed on PFA-fixed, paraffin-embedded hearts (n=3 hearts/group sectioned at 4μm). Sections were deparaffinized, rehydrated and antigen retrieval was induced by heat (100°C for 20 mins) in a citrate buffer (10mM citric acid, 0.05% Tween-20, pH 6.0). Following blocking (10% normal goat serum in 1%BSA/PBS), sections were incubated in vinculin antibody (Sigma Aldrich, clone hVIN-1, V9131, diluted 1:10 in blocking buffer) overnight at 4°C, followed by Cy™3-conjugated AffiniPure goat anti-mouse IgG
(Jackson Laboratories, diluted 1:200 in 1%BSA/PBS). Sections were mounted in Prolong® Gold antifade reagent (Life Technologies) and visualized using an Olympus BX61 microscope.

**Luciferase Reporter Assays**

HEK 293T and H9c2 cells (Australia Cell Bank) were seeded at 40,000 cells/well (passages 2-12) and 30,000 cells/well (passages 10-17), respectively. HEK 293T or H9C2 cells (DMEM, 25 mM, 10% FBS, 1% PS) were transfected with miRNA precursors miR-34a,b,c or unrelated miR controls (miR-Ctrl: miR-210, miR-27a or miR-24-1-5p) at a total concentration of 100nM per well (Life Technologies) in 24-well plates using lipofectamine 2000 (Life Technologies). Pre-validated random sequence miRNA precursor molecules were used as negative controls (miR-Neg Ctrl) at 100nM per well (Life Technologies). The seed/target regions of the vinculin, sema4b, pofut and bcl6 3’UTRs (40-60 nucleotides of the predicted miRNA:mRNA binding region) were cloned into the pLightSwitch renilla luciferase vector by SwitchGear Genomics. 200-300ng of each 3’UTR reporter construct and 100ng of β-galactosidase per well were also co-transfected using lipofectamine 2000. After 48 hr, media was removed and cells were incubated for 30 min at room temperature with LightSwitch luciferase assay reagent containing renilla luciferase substrate and lysis buffer, as per manufacturer’s instructions. Renilla luciferase activity was then measured using a Microlumat Plus luminometer (Berthold). Luciferase activity was normalized by quantifying expression of β-galactosidase using a β-galactosidase detection assay (Promega). Briefly, lysates were incubated with 1×β-gal buffer for 30 min at 37°C and absorption was then measured at 420nm using a microplate spectrophotometer (BioRad). Data is presented as the ratio of 3’UTR luciferase activity to β-galactosidase expression and are representative of four to five independent experiments.
Statistical Analyses

Statistical analyses were performed using StatView (Version 5.0.1, SAS Institute Inc.). Results are presented as mean±SEM. Differences between groups were identified using one-way analysis of variance (ANOVA) followed by Fisher’s post-hoc tests, unless otherwise indicated. Unpaired t-tests were used when comparing two groups for a single measure. A value of $P<0.05$ was considered significant. All relative units are expressed as a fold change with the relevant control group normalized to 1.

References
Legends

SI Figure 1: Up-regulation of miR-34 family members in response to MI. Gene expression of miR-34 family members by microarray (A) and RT-qPCR (B) in hearts from control mice (non-transgenic) subjected to MI or the sham operation. Panel A, N=4 per group. Panel B, N=5 for sham group and N=7 for MI group. *P<0.05 vs sham.

SI Figure 2: Inhibition of miR-34a with LNA-antimiR-34a from 1 day to 2 months. A) Dosing regimen in adult male mice. B) Inhibition of miR-34a relative to snoU6 by RT-qPCR in hearts from mice harvested 1 day, 1 month and 2 months after LNA-antimiR-34a delivery compared with LNA-control. N=3 per group, *P<0.05 vs LNA control.

SI Figure 3: Experimental timeline and dosing regimen for mice subjected to MI or pressure overload. A) MI model and B) pressure overload model (TAC).

SI Figure 4: 15-mer and 8-mer LNA-control dosed mice show comparable morphological and functional parameters. A) Body weight, B) Heart weight / tibia length (HW/TL), C) fractional shortening and D) left ventricular posterior wall thickness (LVPW). 15-mer, N=2 per group; 8-mer N=3 per group. E) Alignment of the 8-mer LNA-control sequence with the 15-mer LNA-control sequence. * common nucleotides.

SI Figure 5: Significant inhibition of miR-34a but not miR-34b and miR-34c with LNA-antimiR-34a. A) Expression of miR-34a, miR-34b and miR-34c relative to snoU6 by RT-qPCR in hearts from mice harvested 2 months after LNA-antimiR-34a delivery compared with LNA-control. *P<0.05 vs LNA control (unpaired t-test). N=3 per group. B) Sequence alignment of mmu-miR-34a, mmu-miR-34b, and mmu-miR-34c. * similar nucleotides between the 3 sequences.
SI Figure 6: Inhibition of miR-34a alone does not improve cardiac function in MI mice.

Fractional shortening in sham and MI mice at comparable heart rates (HR) at 8 weeks post-MI (MI CON, HR: 583±36 bpm; MI antimiR-34, HR: 512±24 bpm; MI antimiR-34a, HR: 536±14 bpm). N=3 for the sham group and MI dosed with LNA-antimiR-34a. N=5 for the MI groups dosed with LNA-control and LNA-antimiR-34. AntimiR delivery at 2 days post-sham/MI (highlighted by arrow). *P<0.05 vs sham LNA-control and the same group at the previous time point(s). †P<0.05 vs MI LNA-control at the same time point. ‡P<0.05 vs MI LNA-antimiR-34 at the same time point.

SI Figure 7: More favorable molecular profile in hearts of LNA-antimiR-34 dosed MI mice and up-regulation of miR-34 targets. RT-qPCR analysis of A) interleukin-6 (Il6) and B) collagen 1α1 (coll1α1) in the infarct zone (left) and remote zone (right) of the MI model dosed with LNA-control (CON), LNA-antimiR-34 (anti-34) or LNA-antimiR-34a (anti-34a). C) aMHC/βMHC ratio, D) cyclin D1 (Ccn1d1), E) Sirt1 and F) Pnus (Ppp1r10) in the remote zone. All standardized to Hprt1. *P<0.05 vs sham, †P<0.05, or as shown (unpaired t-test). N=3-5 per group.

SI Figure 8: LNA-antimiR-34 improves systolic function in mice with established cardiac dysfunction due to TAC. Representative M-mode echocardiograms from mice dosed with LNA-control (CON) or LNA-antimiR-34 five weeks after TAC or sham surgery and followed for 6 weeks post-treatment.

SI Figure 9: Up-regulation of a miR-34 target, Pofut1, in TAC mice treated with LNA-antimiR-34. A) Western blot and B) RT-qPCR analysis of POFUT1/Pofut1 standardized to GAPDH/Hprt1, respectively. N=3-4 for LNA-control TAC mice and N=4-5 for LNA-antimiR-34 TAC mice. *P<0.05 vs LNA-control (CON) dosed TAC mice.
Figure 10: Vinculin, Sema4b, Pofut1 and Bcl6 are experimentally validated targets of miR-34a,b,c. Sequence alignment of miR-34a, -34b, -34c and 3’UTR of Vcl (panel A), Sema4b (panel B), Pofut1 (panel C), and Bcl6 (panel D), and the miR-34a,b,c seed regions (shaded grey) within the respective 3’UTRs showing conservation among species (Mmu-mouse, Rno-rat, Hsa-human and Ptn-pan troglodytes (chimpanzee); N denotes unknown sequence). E) Schematic of plasmid construction with a segment of the 3’UTR cloned downstream of the luciferase-encoding region. F) Luciferase reporter assays for the 3’UTRs of Vcl, Sema4b, Pofut1 or Bcl6 in HEK 293T cells co-transfected with a negative control (miR-Neg Ctrl), miR-34a,b,c or a control oligonucleotide (miR-Ctrl:miR-210, miR-27a, miR-24-1-5p). *P<0.05 relative to controls. Data is presented as the ratio of 3’UTR luciferase activity to β-galactosidase expression and are representative of 4-5 independent experiments.

SI Figure 11: LNA-antimiR-34 induced inhibition of miR-34a, miR-34b and miR-34c in heart, kidney liver and bone, and histological assessment. A) Inhibition of miR-34a, miR-34b and miR-34c relative to snoU6 by RT-qPCR in heart, kidney, liver and bone from control/sham adult mice dosed with LNA-antimiR-34 or LNA-control. For heart, kidney and liver the dosing regimen is shown in SI Fig 3B. For bone, the dosing regimen is shown in SI Fig 3A. N=3-4 per group, *P<0.05 vs LNA control. B) Heart (LV), kidney and liver sections stained with H&E. Scale bar=100μm. Bone, decalcified sections were stained with toluidine blue. Scale bar=25 μm. CB=cortical bone, TB=trabecular bone. Osteoblasts highlighted with red arrow heads. N.B. The white gap between the osteoblasts and CB in the LNA-antimiR-34 dosed mouse is a sectioning artefact.
SI Figure 1

A  Microarray

Gene expression (log$_2$)

<table>
<thead>
<tr>
<th>miR-34a</th>
<th>miR-34b</th>
<th>miR-34c</th>
</tr>
</thead>
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<tr>
<td><strong>8.5</strong></td>
<td><strong>6.5</strong></td>
<td>5.0</td>
</tr>
</tbody>
</table>

Sham  MI

P=0.1

B  RT-qPCR

miR-34a, b, -c/snoU6

Relative expression

<table>
<thead>
<tr>
<th>miR-34a</th>
<th>miR-34b</th>
<th>miR-34c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2.5</strong></td>
<td><strong>2.0</strong></td>
<td>1.5</td>
</tr>
</tbody>
</table>

Sham  MI

P=0.1
SI Figure 2

A  LNA-antimiR-34a

LNA s.c. injections 3 x 25 mg/kg

Harvest tissue Harvest tissue Harvest tissue

Day/month 1 2 3 1 day 1 month 2 months

post subcutaneous injection

B

<table>
<thead>
<tr>
<th>Time</th>
<th>LNA control</th>
<th>LNA antimiR-34a</th>
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<td>1 day</td>
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</tr>
<tr>
<td>1 month</td>
<td>1.0</td>
<td>*</td>
</tr>
<tr>
<td>2 months</td>
<td>1.0</td>
<td>*</td>
</tr>
</tbody>
</table>

Relative expression

mIR-34a / snoU6

post subcutaneous injection
SI Figure 3

A

SHAM / MI Surgery

LNA s.c. injections
3 x 25mg/kg

Harvest tissue

Day 0 2 3 4 8 weeks

B

SHAM / TAC Surgery

LNA s.c. injections
3 per week x 6 weeks

Harvest tissue

Week 0 5 6 11

25 10 10

10 10 10 (mg/kg)
SI Figure 4

A

B

C

D

E

LNA control 8 mer 5’- TCATACTA - 3’
LNA control 15 mer 5’- TCATACTATATGACA - 3’
********
SI Figure 5

A

![Bar graph showing relative expression of miR-34a, miR-34b, and miR-34c with LNA control and LNA antimiR-34a treatments.](image)

B

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<tr>
<th>miRNA</th>
<th>5’ sequence</th>
<th>3’ sequence</th>
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<tr>
<td>mmu-miR-34a</td>
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<tr>
<td>mmu-miR-34b</td>
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<tr>
<td>mmu-miR-34c</td>
<td>AGGCAGUGUAGUUAGCUGAUUGC</td>
<td>3’</td>
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<td>******</td>
</tr>
<tr>
<td></td>
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<td>***</td>
</tr>
</tbody>
</table>
SI Figure 7

A

IL6 / Hprt1

Fold change

P=0.06

SHAM | CON | anti-34 | anti-34a

MI-infarct zone

B

Col1α1 / Hprt1

Fold change

P=0.06

SHAM | CON | anti-34 | anti-34a

MI-infarct zone

C

α-MHC/β-MHC ratio

Fold Change

P=0.07

SHAM | CON | anti-34 | anti-34a

MI-remote zone

D

Cnnd1 / Hprt1

Fold Change

P=0.1

CON | anti-34 | anti-34a

MI-remote zone

E

Sirt1 / Hprt1

Fold Change

P=0.07

CON | anti-34 | anti-34a

MI-remote zone

F

Ppp1r10 / Hprt1

Fold Change

P=0.09

CON | anti-34 | anti-34a

MI-remote zone
SI Figure 8

<table>
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<tr>
<th></th>
<th>Baseline</th>
<th>5 weeks</th>
<th>11 weeks</th>
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<tr>
<td>SHAM CON</td>
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<td></td>
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</tr>
<tr>
<td>SHAM antimiR-34</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TAC CON</td>
<td></td>
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<tr>
<td>TAC antimiR-34</td>
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</table>
**SI Figure 9**

**A**

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<th>POFUT1</th>
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<td><img src="Image2" alt="GAPDH Image" /></td>
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<td>a</td>
<td><img src="Image1" alt="POFUT1 Image" /></td>
<td><img src="Image2" alt="GAPDH Image" /></td>
</tr>
</tbody>
</table>

![Bar Graph](Image3)

POFUT1/GAPDH Fold change

CON | antimiR-34

TAC

P = 0.08

**B**

![Bar Graph](Image4)

Pofut1/Hprt1 Fold Change

CON | antimiR-34

TAC

*
**Figure 10**

**A** Vinclulin

<table>
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<tr>
<th>3'</th>
<th>UGUUGG-GCAUUGCUGAGCAGGU</th>
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<th>mmu-miR-34a</th>
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<tbody>
<tr>
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<td>UUUUAUGCUGCUUUGACUCGCCA</td>
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<td>Vcl 3'UTR</td>
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**B** Sema4b

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<tr>
<th>3'</th>
<th>UGUUGG-GCAUUGCUGAGCAGGU</th>
<th>5'</th>
<th>mmu-miR-34a</th>
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<tr>
<td>5'</td>
<td>AAGGGAAGCU-U-GACUCGCCA</td>
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<td>Sema4b 3'UTR</td>
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**C** Pofut1

<table>
<thead>
<tr>
<th>3'</th>
<th>UGUUGG-GCAUUGCUGAGCAGGU</th>
<th>5'</th>
<th>mmu-miR-34a</th>
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<tbody>
<tr>
<td>5'</td>
<td>ACUGCCAGCCACGUCCACUGCCCA</td>
<td>3'</td>
<td>Pofut1 3'UTR</td>
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**D** Bcl6

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<tr>
<th>3'</th>
<th>UGUUGG-GCAUUGCUGAGCAGGU</th>
<th>5'</th>
<th>mmu-miR-34a</th>
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<tbody>
<tr>
<td>5'</td>
<td>ACUGCCAGCCACGUCCACUGCCCA</td>
<td>3'</td>
<td>Bcl6 3'UTR</td>
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</table>

**Species sequence alignment**

Mmu UGUUGG-GCAUUGCUGAGCAGGU
Rno UGUUGG-GCAUUGCUGAGCAGGU
Hsa UGUUGG-GCAUUGCUGAGCAGGU
Ptr UGUUGG-GCAUUGCUGAGCAGGU

**Relative luciferase activity**

- HEK 293T
- miR-Neg Ctrl
- miR-34abc
- miR-Ctrl

**Fold change**

- Vcl
- Sema4b
- Pofut1
- Bcl6
SI Table 1
Morphological data for C57BL/6 mice subjected to the sham or MI operation, followed by treatment with LNA-control, LNA-antimiR-34 (8-mer) or LNA-antimiR-34a (15-mer) for 8 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>MI</th>
<th>LNA-control</th>
<th>LNA-antimiR-34</th>
<th>LNA-antimiR-34a</th>
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<tr>
<td></td>
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<td>LNA-control 1</td>
<td>LNA-control 2</td>
<td>LNA-antimiR-34 1</td>
<td>LNA-antimiR-34a 1</td>
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<tr>
<td>Infarct size (%)</td>
<td>3</td>
<td>N/A</td>
<td>28.7 ± 1.7*</td>
<td>26.1 ± 2.7*</td>
<td>28.9 ± 3.6*</td>
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<tr>
<td>BW (g)</td>
<td>29.8 ± 0.9</td>
<td>32.4 ± 0.9</td>
<td>32.0 ± 0.4</td>
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<tr>
<td>TL (mm)</td>
<td>16.2 ± 0.1</td>
<td>16.4 ± 0.1</td>
<td>16.4 ± 0.2</td>
<td>16.5 ± 0.1</td>
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<tr>
<td>HW (mg)</td>
<td>146.9 ± 7.7</td>
<td>195.6 ± 5.6*</td>
<td>164.8 ± 5.9†</td>
<td>179.5 ± 8.1*</td>
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<tr>
<td>AW (mg)</td>
<td>7.5 ± 0.2</td>
<td>12.9 ± 0.8*</td>
<td>9.6 ± 0.8</td>
<td>12.3 ± 2.1*</td>
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<tr>
<td>LW (mg)</td>
<td>150.6 ± 6.1</td>
<td>169.1 ± 5.6‡</td>
<td>142.9 ± 7.4†</td>
<td>200.4 ± 31.0‡</td>
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<tr>
<td>RVW (mg)</td>
<td>28.3 ± 1.4</td>
<td>34.6 ± 1.3*</td>
<td>28.9 ± 1.6†</td>
<td>37.2 ± 7.1</td>
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<tr>
<td>LVW (mg)</td>
<td>105.7 ± 7.4</td>
<td>135.2 ± 2.7*</td>
<td>120.6 ± 4.4†</td>
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<td>SW (mg)</td>
<td>83.1 ± 3.9</td>
<td>94.5 ± 7.0</td>
<td>86.4 ± 7.3</td>
<td>89.1 ± 4.3</td>
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<tr>
<td>KW (mg)</td>
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<td>437.5 ± 23.4</td>
<td>394.5 ± 14.5</td>
<td>399.6 ± 29.5</td>
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<tr>
<td>HW/TL (mg/mm)</td>
<td>9.06 ± 0.47</td>
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<tr>
<td>AW/TL (mg/mm)</td>
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<td>0.59 ± 0.05†</td>
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<td>LW/TL (mg/mm)</td>
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<td>10.30 ± 0.32‡</td>
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</tr>
<tr>
<td>LVW/TL (mg/mm)</td>
<td>6.52 ± 0.45</td>
<td>8.24 ± 0.16*</td>
<td>7.35 ± 0.28†</td>
<td>7.46 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>SW/TL (mg/mm)</td>
<td>5.12 ± 0.22</td>
<td>5.76 ± 0.42</td>
<td>5.28 ± 0.50</td>
<td>5.39 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>KW/TL (mg/mm)</td>
<td>25.85 ± 1.18</td>
<td>26.65 ± 1.35</td>
<td>24.01 ± 0.75</td>
<td>24.17 ± 1.76</td>
<td></td>
</tr>
</tbody>
</table>

BW: body weight, TL: tibia length, HW: heart weight, AW: atria weight, LW: lung weight, RVW: right ventricular weight, LVW: left ventricular weight, SW: spleen weight, KW: kidney weight. HW/TL: heart weight/ tibia length ratio, AW/TL: atria weight/ tibia length ratio, LW/TL: lung weight/ tibia length ratio, LVW/TL: left ventricular weight/ tibia length ratio, RVW/TL: right ventricular weight/ tibia length ratio, SW/TL: spleen weight/ tibia length ratio, KW/TL: kidney weight/ tibia length ratio. Data are shown as mean ± SEM. One-way ANOVA followed by Fisher’s post-hoc test. *P<0.05 vs. Sham LNA control, †P<0.05 vs. MI LNA control. ‡P≤0.1 vs Sham LNA control.
**SI Table 2.** Echocardiography data of sham and MI mice at baseline, 2 days post MI and 8 weeks after treatment with either LNA control or LNA antimiR-34.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>2 days post-MI</th>
<th>8 weeks post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham control</td>
<td>MI control</td>
<td>Sham antimiR-34</td>
</tr>
<tr>
<td>No. of animals</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>BW (g)</td>
<td>24.2 ± 0.4</td>
<td>28.3 ± 1.0</td>
<td>27.6 ± 0.6</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>602 ± 24</td>
<td>585 ± 10</td>
<td>585 ± 15</td>
</tr>
<tr>
<td>LVPW, mm</td>
<td>0.80 ± 0.01</td>
<td>0.85 ± 0.02</td>
<td>0.84 ± 0.02</td>
</tr>
<tr>
<td>IVS, mm</td>
<td>0.83 ± 0.02</td>
<td>0.84 ± 0.02</td>
<td>0.83 ± 0.01</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>4.00 ± 0.19</td>
<td>4.02 ± 0.12</td>
<td>3.84 ± 0.15</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.41 ± 0.15</td>
<td>2.41 ± 0.10</td>
<td>2.37 ± 0.07</td>
</tr>
<tr>
<td>FS, %</td>
<td>40 ± 1</td>
<td>40 ± 1</td>
<td>38 ± 2</td>
</tr>
</tbody>
</table>

BW, body weight; LV, left ventricular; LVPW, LV posterior wall thickness; IVS, interventricular septum thickness; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; FS, fractional shortening. Data are shown as mean ± SEM. One way ANOVA followed by Fisher’s Post hoc Test. *P<0.05 vs baseline of same group, †P<0.05 vs Sham LNA control at same time point, ‡P<0.05 vs MI LNA control at same time point, §P<0.05 vs same MI group at 2 days post-MI, ¶P=0.064 vs MI LNA control at 8 weeks, ¶¶P=0.076 vs Sham LNA control at the same time point.
**SI Table 3.** Echocardiography data of sham and TAC mice at baseline, 5 weeks post TAC and 6 weeks after treatment with either LNA-control or LNA antimiR-34.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>5 weeks post-TAC</th>
<th>6 weeks post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham control</td>
<td>TAC antimiR-34</td>
<td>Sham control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>control antimiR-34</td>
<td>TAC control antimiR-34</td>
</tr>
<tr>
<td>Number</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Number</td>
<td>6</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Number</td>
<td>6</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>BW (g)</td>
<td>27.6±0.2</td>
<td>25.9±1.6</td>
<td>30.1±0.7</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>646±58</td>
<td>614±35</td>
<td>604±26</td>
</tr>
<tr>
<td>LVPW</td>
<td>0.88±0.02</td>
<td>0.91±0.01</td>
<td>0.89±0.01</td>
</tr>
<tr>
<td>IVS</td>
<td>0.89±0.02</td>
<td>0.90±0.01</td>
<td>0.89±0.03</td>
</tr>
<tr>
<td>LVEDD</td>
<td>3.72±0.11</td>
<td>3.77±0.06</td>
<td>3.79±0.09</td>
</tr>
<tr>
<td>LVESD</td>
<td>2.20±0.10</td>
<td>2.26±0.08</td>
<td>2.25±0.08</td>
</tr>
<tr>
<td>FS, %</td>
<td>41±1</td>
<td>40±3</td>
<td>41±1</td>
</tr>
</tbody>
</table>

BW, body weight; HR, heart rate; LV, left ventricular; LVPW, LV posterior wall thickness; IVS, interventricular septum thickness; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; FS, fractional shortening. LVPW, IVS, LVEDD and LVESD presented in mm. Data are shown as mean ± SEM. One way ANOVA followed by Fisher’s Post hoc Test. *P<0.05 vs baseline of same group; †P<0.05 vs sham of same group at same time point; ‡P<0.05 vs TAC LNA control 5 weeks post TAC; §P<0.05 vs TAC LNA control 6 weeks post treatment; ¶P<0.05 vs TAC LNA antimiR-34 5 weeks post-TAC; || P<0.05 vs Sham LNA control 6 weeks post treatment.
**SI Table 4.** Morphological data for sham and TAC mice following five weeks of pressure overload and six weeks of treatment with LNA-control or LNA-antimiR-34.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LNA-control</td>
<td>LNA-antimiR-34</td>
</tr>
<tr>
<td><strong>No. of animals</strong></td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>31.5 ± 0.4</td>
<td>30.9 ± 1.4</td>
</tr>
<tr>
<td><strong>TL (mm)</strong></td>
<td>16.6 ± 0.1</td>
<td>16.5 ± 0.2</td>
</tr>
<tr>
<td><strong>HW (mg)</strong></td>
<td>128.1 ± 1.5</td>
<td>128.3 ± 5.6</td>
</tr>
<tr>
<td><strong>AW (mg)</strong></td>
<td>8.3 ± 0.3</td>
<td>8.8 ± 0.7</td>
</tr>
<tr>
<td><strong>LW (mg)</strong></td>
<td>135.3 ± 3.0</td>
<td>132.2 ± 3.3</td>
</tr>
<tr>
<td><strong>HW/TL (mg/mm)</strong></td>
<td>7.73 ± 0.14</td>
<td>7.78 ± 0.24</td>
</tr>
<tr>
<td><strong>AW/TL (mg/mm)</strong></td>
<td>0.50 ± 0.02</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td><strong>LW/TL (mg/mm)</strong></td>
<td>8.17 ± 0.20</td>
<td>8.03 ± 0.16</td>
</tr>
<tr>
<td><strong>No. of animals</strong></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>KW (g)</strong></td>
<td>436.9 ± 23.2</td>
<td>402.2 ± 37.3</td>
</tr>
<tr>
<td><strong>KW/TL (mg/mm)</strong></td>
<td>26.28 ± 1.16</td>
<td>24.88 ± 1.75</td>
</tr>
</tbody>
</table>

BW: body weight, HW: heart weight, AW: atria weight, LW: lung weight, TL: tibia length, KW: kidney weight. HW/TL: heart weight/ tibia length ratio, AW/TL: atria weight/ tibia length ratio, LW/TL: lung weight/ tibia length ratio, KW/TL: kidney weight/ tibia length ratio. Data are shown as mean ± SEM. One-way ANOVA followed by Fisher’s post-hoc test. *P<0.05 vs. sham of the same treatment group, †P<0.05 vs. TAC LNA-control, ‡P=0.05 vs. TAC LNA-control.