MiR-378 Controls Cardiac Hypertrophy by Combined Repression of Mitogen-Activated Protein Kinase Pathway Factors

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Background—Several microRNAs (miRs) have been shown to regulate gene expression in the heart, and dysregulation of their expression has been linked to cardiac disease. miR-378 is strongly expressed in the mammalian heart but so far has been studied predominantly in cancer, in which it regulates cell survival and tumor growth.

Methods and Results—Here, we report tight control of cardiomyocyte hypertrophy through miR-378. In isolated primary cardiomyocytes, miR-378 was found to be both necessary and sufficient to repress cardiomyocyte hypertrophy. Bioinformatic prediction suggested that factors of the mitogen-activated protein kinase (MAPK) pathway are enriched among miR-378 targets. Using mRNA and protein expression analysis along with luciferase assays, we validated 4 key components of the MAPK pathway as targets of miR-378: MAPK1 itself, insulin-like growth factor receptor 1, growth factor receptor-bound protein 2, and kinase suppressor of ras 1. RNA interference with these targets prevented the prohypertrophic effect of anti-miR-378, suggesting their functional relation with miR-378. Because miR-378 significantly decreases in cardiac disease, we sought to compensate for its loss through adeno-associated virus–mediated, cardiomyocyte-targeted expression of miR-378 in an in vivo model of cardiac hypertrophy (pressure overload by thoracic aortic constriction). Restoration of miR-378 levels significantly attenuated thoracic aortic constriction–induced cardiac hypertrophy and improved cardiac function.

Conclusions—Our data identify miR-378 as a regulator of cardiomyocyte hypertrophy, which exerts its activity by suppressing the MAPK signaling pathway on several distinct levels. Restoration of disease-associated loss of miR-378 through cardiomyocyte-targeted adeno-associated virus–miR-378 may prove to be an effective therapeutic strategy in myocardial disease. (Circulation. 2013;127:2097–2106.)

Key Words: cardiomyocytes ■ gene therapy ■ hypertrophy ■ microRNA

MicroRNAs (miRs) are small, noncoding RNAs that post-transcriptionally regulate gene expression.1 MiRs have been implicated in a wide variety of physiological processes, and individual miRs are known to participate in diseases, including cardiovascular disease.2 Still, the vast majority of miRs encoded by the human genome are not understood with respect to their function.

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We recently developed a method to screen libraries of multiple synthetic miRs for the induction of cardiomyocyte hypertrophy, a hallmark of the myocardial stress response.3 This approach not only validated previously known hypertrophy-regulating miRs but also helped to identify novel prohypertrophic and antihypertrophic miRs.3 In this context, our assay revealed first evidence that miR-378-3p (previously annotated as miR-422b) acts as an antihypertrophic miR. Interestingly, this miR also appeared in a complementary approach in which we searched for miRs that are strongly expressed in the heart based on the presumption that strong tissue-specific expression correlates with physiological relevance. MiR-378-3p proved to be among the most prominent miRs in adult rat cardiomyocytes.3 Together, these findings prompted further functional characterization of this miR.
PBS was added. Two days after stimulation, protein lysates were prepared for Western blot analysis. A similar procedure was followed for transfection of antimiR-378 (50 nmol/L, Exiqon) and antimiR control (50 nmol/L, Exiqon).

**Immunostaining and Apoptosis Detection**

Immunostaining of NRCMs in a 96-well format and automated cell size measurement were performed as described. Apoptosis was analyzed in isolated NRCMs or cross sections of paraffin-preserved mouse hearts by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) as described, with the in situ cell death detection kit (Fluorescein, Roche) applied according to the manufacturer’s instructions.

**Animal Disease Models and AAV-Based Therapeutic Intervention**

Thoracic aortic constriction (TAC) was performed on 8-week-old male C57BL/6 N mice (Charles River Laboratories) essentially as described previously. In sham surgery, only the chest was opened, but no ligation of the aorta was carried out. Cardiac dimensions and function were analyzed by pulse-wave Doppler echocardiography before TAC/sham surgery and before the animals were euthanized. β1-adrenergic receptor transgenic mice were analyzed at the age of 12 months, as described previously. For cardiotoxic expression of exogenous miR-378, 5-week-old wild-type male mice received AAV9–miR-378 (1×10^{12} genome copies per mouse) or PBS by tail vein injection, and TAC was performed 3 weeks later. All animal studies were performed in accordance with the relevant guidelines and regulations of the responsible authorities.

**Statistical Analysis**

Data are shown as means±SEM. Statistical analysis was performed with Prism (GraphPad Software). The Student t test or 2-way ANOVA followed by the Bonferroni test was used as appropriate (see figure legends and Methods in the online-only Data Supplement).

**Results**

**Identification of miR-378 as an Antihypertrophic miR**

This study was initiated by 2 parallel approaches to identify miRs with cardiac function: our multwell screen for morphological changes in isolated NRCMs on transfection with synthetic miRs and quantitative analysis of miR expression levels in the myocardium.

Transfection of miR-378 affected NRCM size only marginally under basal conditions but largely prevented phenylephrine-induced cardiomyocyte hypertrophy (Figure 1A). Phenylephrine-treated NRCMs exhibited a reduction in hypertrophy marker miRNAs (Nppa, Nppb) after transfection with miR-378 (Figure 1C). In line with another report, we also observed lower cell densities in miR-378–transfected cultures as a result of increased apoptosis (see Figure I in the online-only Data Supplement). Consistently, antimiR-378 interfered with the antihypertrophic function of miR-378, leading to enlarged cardiomyocytes (Figure 1B) and increased cell numbers, the latter accompanied by reduced apoptosis (Figure I in the online-only Data Supplement).

**miR-378 is strongly expressed in the rodent heart, where it is 1 of the 50 most prominent miRs in cardiomyocytes. We found expression of miR-378a-3p to account for the vast majority of miR-378–related sequences in mouse; expression of its opposite strand (called miR-378-5p or miR-378*) is far lower (Figure VIB in the online-only Data Supplement).
MiR-378 in cardiomyocytes exceeded that of cardiac fibroblasts at least 10-fold (Figure 1D). Cardiac expression of miR-378 undergoes a boost during mouse ontogenesis (Figure II in the online-only Data Supplement and Reference 12), thus raising the question of whether cardiac disease conditions would induce a return of miR-378 expression to fetal levels, as we have observed for other miRs.16 We chose 2 disease models: TAC as a model for chronic pressure overload and transgenic overexpression of the β1-adrenergic receptor, which leads to severe heart failure.16 Remarkably, expression of miR-378a-3p in diseased hearts was downregulated by 54% and 42%, respectively (Figure 1E). In line with this, left ventricular myocardium from patients with dilated cardiomyopathy revealed a decrease in miR-378 expression (Figure 1E), suggesting that this miR is involved in the development of cardiac disease.

MiR-378 Targets Key Components of the MAPK Pathway

We next investigated which mRNA targets are controlled by miR-378 and used miTALOS,19 a recently developed bioinformatic tool that predicts functional association of miRs with signaling pathways. A query with human miR-378 yielded the highest enrichment of targets within the MAPK signaling pathway. These targets are MAPK1 (also called extracellular regulated kinase 2), kinase suppressor of ras 1 (KSR1), growth factor receptor-bound protein 2 (GRB2), and IGF1R. Consistent with the prediction through miTALOS, TargetScan (version 6.2) identified binding sites for miR-378 in the 3′ untranslated regions of these mRNAs that are conserved in evolution between humans and rodents. Next, we isolated mRNA from NRCMs that had been transfected with miR-ctrl or miR-378 and subjected it to microarray analysis. The results (Figure 2A) show that, among few others, the levels of Grb2 and Ksr1 mRNAs were reduced in miR-378–transfected cells, whereas Igf1r and Mapk1 mRNAs were unaltered (Figure 2A). Validation of the microarray data by qualitative polymerase chain reaction showed repression of each of the 4 potential targets by miR-378 (Figure 2B).

Similarly, immunoblot detection in NRCM lysates confirmed lower amounts of GRB2, IGF1R, KSR1, and MAPK1; the reciprocal observation was made in antimiR-378 transfectants (Figure 2C). Elevated miR-378 levels or inhibition of miR-378 altered not only target expression but consequently also MAPK pathway activation. Indeed, Western blotting with an antibody against phosphorylated MAPK1 and MAPK3 revealed a decrease in the signal in miR-378 transfectants, whereas antimiR-378 induced higher levels of phosphorylated MAPK (Figure 2C), consistent with a functional link between miR-378 and the activity of MAPK1/3.

To test for direct and specific regulation of the presumed mRNA targets by miR-378, we generated luciferase reporter constructs in which firefly luciferase cDNA was linked to the
untranslated region of the respective targets. NRCMs were transfected with each of the reporter constructs, together with miR-378 or miR-ctrl, and suppression of the luciferase signal was quantified after cell lysis. As an internal control for transfection, the reporter construct also comprised Renilla luciferase cDNA under an autonomous promoter, and data were assessed as the ratio of both luciferase signals. Indeed, miR-378 efficiently reduced firefly luciferase luminescence for all 4 targets, whereas miR-ctrl and antimiR-378 conferred the opposite (Figure 3A). Mutation of seed binding sites for miR-378 made the reporter insensitive to miR-378 or antimiR-378 transfection (Figure 3A), indicating the specific dependence of target 3′ untranslated regions on miR-378. Taken together, the data obtained thus far indicate that miR-378 regulates 4 components of the MAPK pathway in cardiomyocytes, namely MAPK1, GRB2, KSR1, and IGF1R.

Functional Relevance of miR-378 Targets

Despite the existence of conserved binding sites for miR-378 in GRB2, IGF1R, KSR1, and MAPK1 and our ability to validate binding of miR-378 to their respective 3′ untranslated regions, the question remained whether a functional relationship between miR-378 and these targets existed with respect to hypertrophy. On the basis of the assumption that antimiR-378 exerts its prohypertrophic effect through the derepression of specific targets, we designed an siRNA-based strategy (Figure 3B) to validate the functional relevance of those targets. If the targets we identified were responsible for the effect of antimiR-378 on NRCMs, then RNA interference with their expression (by siRNA) should specifically counteract antimiR-378–induced hypertrophy. This concept was tested by transfecting cardiomyocytes in vitro with siRNAs to silence the expression of targets of miR-378 within the MAPK pathway. One day after isolation, NRCMs were transfected with antimiR-378 or siRNA, followed by a switch to a low-serum medium. Phenylephrine was added, and after 2 days of incubation, cell size was measured. Consistent with our earlier observations (Figure 1B), antimiR-378 alone enhanced phenylephrine-induced hypertrophy, yet the siRNA against Mapk1 efficiently prevented the prohypertrophic effect of antimiR-378 (Figure 3C). Likewise, the knockdown of Grb2, Ksr1, and Igf1r attenuated the prohypertrophic effect of antimiR-378 (Figure 3C).

Cardiomyocyte-Targeted Expression of miR-378 In Vivo Partially Prevents Cardiac Hypertrophy

We then assessed in vivo whether TAC-induced downregulation of miR-378 contributed to the cardiac phenotype and whether
exogenous miR-378 could prevent this. We used AAV9 because it shows strong tropism for the heart, particularly for cardiomyocytes. 20,21 Five-week-old mice were infected with AAV9–miR-378 or with an equivalent volume of PBS (control), and 3 weeks later, they were subjected to TAC or sham surgery (Figure 4A). After 3 more weeks, echocardiography was carried out, and tissue samples were collected for further analysis (Figure 4A). In confirmation of our initial observations, the noninfected controls showed decreased miR-378 expression on TAC treatment (Figure 4B). Cardiac expression of miR-378 rose by a factor of \( \approx 2.5 \) in sham-treated mice infected with AAV9–miR-378 compared with controls, indicative of continuous miR expression until the end of the experiment (Figure 4B). MiR-378 levels in the TAC-treated group infected with AAV9–miR-378 were similar to the amount detected in noninfected animals under basal conditions (Figure 4B). Two other miRs with known antihypertrophic function, miR-1 and miR-133a,22 showed the expected downregulation under TAC, but their expression was not significantly influenced by AAV9–miR-378 (Figure VIB in the online-only Data Supplement).
We then asked whether compensation with exogenous miR-378 can attenuate the development of structural and functional changes that result from pressure overload of the myocardium. Within the TAC group, administration of AAV9–miR-378 partially prevented left ventricular hypertrophy and improved cardiac function as judged by the ratio of left ventricular weight to tibia length (Figure 4C) and echocardiographic data on left ventricular mass, left ventricular volume, chamber diameter, ejection fraction, and fractional shortening (Figure 4E). Consistently, mRNA levels of the hypertrophy markers Nppa, Nppb, and Myh7 were reduced in the AAV9 prevention group (Figure VIA in the online-only Data Supplement). The
development of pulmonary congestion (ratio of lung weight to tibia length) as an indirect indicator of cardiac function was also significantly lowered by AAV9–miR-378 (Figure 4D). Importantly, the antihypertrophic effect is not an intrinsic property of AAV9, as demonstrated by the finding that TAC-induced hypertrophy parameters after infection with AAV9–green fluorescent protein were almost identical to the PBS control (Figure V in the online-only Data Supplement).

Although miR-378 is a cardiomyocyte-specific miR (Figure 1D), this does not exclude the possibility of an indirect influence on cardiac fibroblast activity through, for example, paracrine factors. Indeed, AAV9-mediated compensation of miR-378 downregulation prevented interstitial fibrosis in left ventricular myocardium after TAC (Figure 4F and 4G). To determine whether the decrease in left ventricular weight (Figure 4C) was indeed caused by inhibition of cardiomyocyte hypertrophy, we analyzed regions of the subendocardial myocardium by morphometric analysis. Automated image processing of wheat-germ agglutinin– and SYTOX Green–stained sections allowed us to select for cardiomyocytes, which were sectioned in the short axis

Given the proapoptotic activity of miR-378 on cardiomyocytes in vitro (and its conflicting interpretation; see the Discussion), we tested whether miR-378 mediates cardiomyocyte apoptosis in vivo. TUNEL staining on whole-heart samples did not reveal a significant change in cardiomyocyte apoptosis with AAV9–miR-378 either under control conditions or after TAC (Figure IC in the online-only Data Supplement). This finding is particularly in good agreement with the improved cardiac function and the partial prevention of myocardial remodeling seen in TAC mice on restoration of miR-378 levels. Together, the experiments carried out in vivo confirmed our findings from cultured NRMCs that miR-378 significantly reduces the extent of cardiac hypertrophy and rule out a significant contribution to apoptosis in the heart under such conditions.

Finally, we tested whether maintenance of physiological miR-378 levels by AAV9–miR-378 affected the expression of prohypertrophic targets. Western blot and qualitative polymerase chain reaction analysis of miR-378 targets revealed a marked upregulation of the 4 MAPK pathway targets of this miR after TAC. This upregulation was significantly prevented by AAV–miR-378 for 3 of these targets (ie, KSR1, MAPK1 and Igf1r) with a trend toward repression observed for Grb2 (Figure 5A).

In summary, this study not only demonstrated the antihypertrophic activity of miR-378 in cardiomyocytes but also provided insight into functional targets of this miR within the MAPKs signaling pathway. MiR-378 appears to target this key regulatory pathway at 4 different levels and thereby effectively controls cardiomyocyte hypertrophy (Figure 5B). Furthermore, our data demonstrate in vivo the cardioprotective potential that therapeutic restoration of miR-378 levels may have in cardiac disease.

Discussion

In this study, we present comprehensive evidence for an antihypertrophic activity of miR-378 in the heart. We demonstrate in vitro and in vivo that miR-378 confers an antihypertrophic phenotype, and we identify and validate GRB2, IGF1R, KSR1, and MAPK1 as targets of this miR.

An important aspect of our study is the verification of miR-378 activity in vivo in a mouse model for chronic pressure overload (TAC). Downregulated expression of miR-378 in the TAC model, together with its restoration by AAV9–mediated exogenous expression, allowed us to elucidate the role of miR-378 in cardiac hypertrophy. The levels of miR-378 achieved with AAV9–miR-378 are nearly physiological (and much lower than what is typically achieved by transfecting cells in vitro with synthetic miR-378). Our in vivo experiments indicated that compensatory expression of miR-378 does not trigger apoptosis in the myocardium (Figure IC in the online-only Data Supplement), despite its proapoptotic effect after transfection of cultured cardiomyocytes with this miR (see also Figure IA in the online-only Data Supplement and Reference 12). In addition, isolated primary myocytes in general have been reported to be very sensitive toward proapoptotic stimuli, with exceedingly high rates of apoptosis not seen in vivo.23 We speculate that this is the reason why antimiR-378–treated cells also exhibited some reduction in apoptosis (Figure IB in the online-only Data Supplement) under apoptosis-promoting conditions (ie, absence of serum or phenylephrine). In line with this, application of AAV9–miR-378 in vivo left the heart largely unaffected under basal conditions (Figure 4C).

As a key target of miR-378, we identified MAPK1 (also called extracellular regulated kinase 2), which, together with MAPK3 (extracellular regulated kinase 1), acts as a signal transduction node that integrates various upstream signals into a few uniform cellular responses such as proliferation, inhibition of apoptosis, and cell growth.24 Despite recent progress from mouse knockout lines, there is vivid discussion about the specific contribution of MAPK1/3 to these cellular responses and the question of whether other kinases like p38 MAPK/Jun N-terminal kinase 1 perform some of the functions previously assigned to MAPK3 and MAPK1.25,26 Our present study cannot ultimately resolve this issue, but it may shed new light on the cardiac role of MAPK1. We provide several lines of evidence that miR-378 directly represses MAPK1 (not MAPK3), a regulation that becomes less tight under pressure overload, when miR-378 is itself downregulated.

Perhaps the most intriguing result of our study is the finding that miR-378 targets 3 additional factors that promote cardiomyocyte hypertrophy and that are in functional context with MAPK1. The relation between these targets and miR-378 was validated by luciferase reporter assays and by the finding that their siRNA-mediated knockdown overrules the contribution of antimiR-378 to cardiomyocyte hypertrophy. KSR1 is a scaffold protein on which MAPK1 depends for activation.24 So far, little is known about a cardiac function of KSR1. Mice deficient in KSR127 did not show developmental defects, but whether reduced levels of KSR1 make this mouse more resistant to cardiac hypertrophy in a disease model needs to be investigated. Interestingly, and in line with our results, a protein to which KSR1 is presumably linked, called isoform A of the Ras association domain-containing protein 1,28,29 was shown to regulate cardiac hypertrophy.30 A prohypertrophic role of GRB2 is well established on the
basis of data from haploinsufficient or transgenic mice. Probably the best-studied miR-378 target with respect to cardiac hypertrophy is IGF1R. As mentioned, regulation of IGF1R by miR-378 was recently shown in vitro and is now supported by our study in vivo. It is unclear whether IGF1R promotes only the physiological form of cardiac hypertrophy because cardiac overexpression of its ligand IGF1 also caused malignant cardiac hypertrophy over time. Because IGF1R activates PI3 kinase/AKT and MAPK1/3 signaling, miR-378 could also indirectly interfere with AKT signaling. Interestingly, genetic knockout of AKT1 or AKT2 in mice indicates that the individual activities of these main cardiac isoforms protect the heart from excessive hypertrophy and apoptosis in the early phase of cardiac stress (as opposed to pathological hypertrophy promoted by MAPK). It is thus tempting to presume that miR-378 balances the adaptive and maladaptive features of AKT and MAPK pathways against each other.

Is combined repression of several mRNAs by miR-378 necessary to efficiently prevent hypertrophy, or would regulation of only 1 target suffice? The answer may lie in the fact that most pathways are interconnected, and thus a phenotypic output may occur if only miRs hit a pathway at various levels. Nonetheless, experimental support of this hypothesis is underrepresented. The miR-17-92 genomic cluster of 6 miRs was shown to collectively regulate the transforming growth factor-β pathway. With some limitations, miR-24 may be exemplary for a single miR with multiple activities in one context, yet the absence of miR-24 seed sequences in its suspected targets leaves the mode of action unresolved. Thus, to the best of our knowledge, our study on the cardiac role of miR-378 is the first experimental support that a single miR targets functionally linked mRNAs within a signaling pathway.

With regard to cardiac hypertrophy, 2 other miRs, miR-1 and miR-133a, deserve attention because both have been suggested to be antihypertrophic. It is thus attractive to speculate that they strengthen the antihypertrophic effect of miR-378. This idea is supported by the finding that miR-1 inhibits IGF1-mediated signaling, apparently by regulating the mRNAs encoding IGF1 or IGF1R. Although mice infected with AAV9–miR-378 do not show elevated cardiac levels of miR-1 or miR-133a (Figure VIB in the online-only Data Supplement), this does not preclude an antihypertrophic potential of these miRs.

Whether and how miR-378 affects apoptosis in the heart is currently controversial because we and others observed increased apoptosis after transfection of cardiomyocytes with miR-378, whereas protection of H9c2 cells from hypoxia-induced apoptosis was reported by others. The latter was attributed to repression of caspase-3, a putative target of miR-378. It is also interesting that miR-378 is highly upregulated in tumors (as opposed to its downregulation in the diseased heart), and it was suggested to have an antiapoptotic effect next to other activities. Not disregarding a role of miR-378 in apoptosis, we believe the most plausible explanation for such discrepant observations lies in the cell types and experimental conditions, ie, the separation from the tissue context, which may increase the rate of cell death. Our finding that in vivo miR-378 does not appear to affect cardiomyocyte apoptosis thus clearly distinguishes its activity in the heart from that in tumors.

Recently, the opposite strand of miR-378, called miR-378*, or miR-378-5p, was functionally characterized on the basis of a miR-378 knockout in mice. These mice displayed elevated mitochondrial fatty acid metabolism and energy expenditure, which correlates with 2 targets of miR-378 and miR-378*. Figure 5. Cardiotropic expression of miR-378 restores mitogen-activated protein kinase (MAPK)-1 signaling and reduces cardiac dysfunction. A, Western blot and quantitative polymerase chain reaction analysis of miR-378 target levels in the myocardium of mice that had undergone thoracic aortic constriction (TAC) and adenovirus–miR-378 treatment. Top, Representative Western blots depicting the expression of miR-378 target proteins. Heat shock protein 90 (HSP90) served as a loading control. Bottom, Quantitative analysis of results (n=5–7; 2-way ANOVA/Bonferroni). B, Scheme depicting the control of the MAPK signaling pathway through miR-378. Statistical significance is shown as *P<0.05, **P<0.01.
called carnitine-O-acetyltransferase (CRAT) and mediator complex subunit 13, respectively. Although the knockout mice do not show a cardiac phenotype under basal conditions, it would be interesting to see whether cardiac remodeling or function is altered under cardiac stress. We observed reduced cardiac levels of CRAT and mediator complex subunit 13 in response to TAC (Figure VIC in the online-only Data Supplement), albeit in the case of CRAT, the opposite was expected, given that miR-378 is itself downregulated in TAC. Thus, a correlation between CRAT, mediator complex subunit 13, and miR-378 is not evident in the heart.

What conclusions can be drawn from our study with respect to therapeutic options? Remodeling of the heart under chronic stress appears to result, at least in part, from downregulation of miR-378 and thus from insufficient control by this miR. Restoration of its expression with the use of AA V9 exerted a cardioprotective potential that may form the basis of future therapeutic approaches.

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While this paper was in review, complimentary data describing an antihypertrophic role of miR-378 in cardiomyocytes in vitro were published (Nagalingam RS, Sundaresan NR, Gupta MP, Geenen D, Solaro RJ, Gupta M. A cardiac enriched microRNA, miR-378 blocks cardiac hypertrophy by targeting Ras-signaling. The Journal of Biological Chemistry. 2013;288:11216–11232).

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disclosures

None.

References

CLINICAL PERSPECTIVE

MicroRNAs (miRs) are small, noncoding RNAs that posttranscriptionally regulate gene expression. Thus, miRs have been shown to regulate many processes in health and disease, including cardiovascular disease. Using a method to screen libraries of multiple synthetic miRs for the induction of cardiomyocyte hypertrophy, a hallmark of the myocardial stress response, we found that the first evidence for an antihypertrophic activity of miR-378 in the myocardium. Subsequent analyses showed that miR-378 repression results in hypertrophic signaling at 4 levels within the mitogen-activated protein kinase signaling pathway. miR-378 was found to be downregulated both in animal models of myocardial disease and in human failing myocardium. Compensation for miR-378 downregulation in a cardiac disease model using viral gene transfer in vivo protected the heart against hypertrophy and dysfunction. Together, these data indicate effective interference of miR-378 with a key hypertrophic signaling pathway. We imagine that targeted delivery of miR-378 to the heart may prove to be an effective therapeutic strategy in myocardial disease.
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SUPPLEMENTAL MATERIAL

miR-378 controls cardiac hypertrophy by combined repression of MAP kinase pathway factors by Ganesan et al.

Supplemental methods

Quantification of miR-378 and mRNA in isolated cells or tissue

RNA was extracted from neonatal rat cardiomyocytes using TriFast (peqLab) and oligoT-primed cDNA synthesis was performed with Superscript II (Invitrogen), both according to the manufacturer’s instructions. Quantitative real time PCR amplification of potential miR-378 targets and hypertrophic markers was performed with primers listed in Supplemental Table 1, using SYBR Green and Platinum Taq polymerase (Invitrogen). Expression of miR-378 in tissue or isolated cells was quantified by Taqman microRNA assays (Applied Biosystems) following manufacturer’s instructions, with primers and taqman probes specific for hsa-miR-378a-3p (miR-378), hsa-miR-378-5p (miR-378*), hsa-miR-1, hsa-miR-133a. Data were normalized to U6 snRNA. MiRNA expression in murine tissue was analyzed using organs (from 6 months old mice), hearts from β1AR-transgenic mice (10 months), or hearts from TAC-treated mice (11 weeks old). Material from wildtype or sham-operated littersmates served as control. RNA isolated from the hearts of these mice (250 ng each) was also used for comprehensive microRNA expression profiling by a service provider (Exiqon). Expression of miR-378 in human heart was analyzed using heart biopsies from patients suffering from dilated cardiomyopathy and non failing control biopsies.

Western blot analysis

Protein extraction was performed in lysis buffer containing protease and phosphatase inhibitors (Roche) and Western blotting was performed using standard procedures. Protein lysates were electrophoresed on 10% or 12% SDS-PAGE gels, transferred onto a PVDF membrane and blocked with BSA for two hours at RT. For Western blot, the following antibodies were used: anti-KSR1 (#611576, BD Biosciences), anti-MAPK1/3 (#9102, Cell Signaling), anti-PhosphoMAPK1/3 (#9101, Cell Signaling), anti-IGF1R (#3027, Cell Signaling), anti-GRB2 (#3972s, Cell Signaling). A monoclonal anti-heat shock protein 90 (HSP90, sc-13119, Santa-Cruz) was used as a loading control. For detection of phospho-MAPK1/3, cells were stimulated for 10 min with 10 μM PE before lysis.
mRNA expression profiling

Microarray analysis of mRNA expression was performed by a service provider (DNAVision SA, Belgium), using Affymetrix technology. 100 ng total RNA isolated from miR-transfected, PE-treated NRCM (see above) was used for analysis.

Knockdown of miR-378 targets

Synthetic siRNAs (ON-TARGETplus SMARTpool, Dharmacon) were used to knockdown each of the miR-378 targets. A non targeting control pool (ON-TARGETplus Control Pool, Dharmacon) was used as control siRNA. For the functional assay, NRCM were plated onto optically optimised 96 well plates and cultured for 24 hours in MEM/5% FCS at 37°C and 1% CO₂. Cells were then transfected with 100nM of the respective siRNAs and/or 50nM antimiR-378 and antimiR-ctrl, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The medium was changed the next day to MEM/0.1% FCS. 48 h after transfection, cells were stimulated with PE to induce hypertrophy and, after another 48 h, immunostaining and automated cell size measurements were performed as described previously (1).

Luciferase reporter assay

200-300 base pairs of the 3’ untranslated region (3’ UTR) of target mRNAs, including the miR-378 binding site therein, were amplified by PCR and cloned as described (2) into a pmiR-RL-TK2 vector, downstream of the sequence which encodes firefly luciferase. Mutation of miR-378 binding sites was carried out with primers carrying the nucleotide exchanges shown in Supplementary Fig. S3, using the QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). Plasmids (150 ng) were co-transfected into NRCM with miR-378 (25 nM, Ambion) or antimiR-378 (12.5 nM, Exiqon) in a 96 well plate and the luciferase activity measured 24h later using the Dual Glo luciferase assay (Promega).

Generation of AAV vectors

Recombinant AAV-miR-378 and AAV-GFP vectors used in this study were prepared by the AAV Vector Unit at the International Centre for Genetic Engineering and Biotechnology Trieste (http://www.icgeb.org/avu-core-facility.html), as described previously (3) with a few modifications. Briefly, infectious recombinant AAV vector particles were generated in HEK293T cells by a cross-packaging approach whereby the vector genome was packaged into AAV capsid serotype-9 (4). Viral stocks were obtained by CsCl₂ gradient centrifugation; rAAV titers, determined by measuring the copy number of viral genomes in pooled, dialyzed gradient fractions, as described previously (5) were in the range of 1x10¹² to 1x10¹³ genome copies per milliliter. For the AAV-miR-378 preparation, the genomic pre-miR sequence was subcloned into the vector plasmid derived from pAAV-MCS (Agilent Technologies) containing a modification in the multiple cloning site to match the insert ends. In all the vectors used in
this study, the transgene is expressed under the transcriptional control of the CMV immediate early promoter.

**Histochemical and immunohistochemical analyses**

For the analysis of collagen deposition, paraffin sections of the left ventricular myocardium were stained with Sirius red and Fast green (6). Collagen content was calculated as the percentage of the area in each section that was stained with Sirius red. For analysis of cardiomyocyte hypertrophy, tissue sections (6 µm) of left ventricular myocardium were stained with SYTOX Green (Life Technologies) for staining of cell nuclei and ALEXA 647-labelled wheat-germ agglutinin (WGA, Invitrogen) for determination of myocyte cross-sectional areas. Individual cells were analyzed in an automated manner. Confocal images were taken at 20x magnification at areas of transversely cut muscle fibers. Common morphology filters were used in the MetaMorph software (Molecular Devices, Sunnyvale, USA) to draw lines separating individual cells based on the WGA staining. Thresholding was applied to exclude regions of background (no cells) or extensive fibrosis. Nuclei were extracted from the green channel. Using morphology filters nuclei that were touching any cell borders were automatically excluded. The remaining nuclei were used to select for cardiomyocytes with centralized nuclei. Cells with a width of less than 7 µm or an area of less than 50 µm were considered non-myocytes and excluded from the analysis. Averages for area and breadth (width of the object perpendicular to the longest chord) of analyzed cells (>20 cells per section) were exported using the MetaMorph integrated morphometry analysis function.

**Statistical analysis**

Data were analyzed for normality using Shapiro-Wilk or Kolmogorov-Smirnov test. Testing for common variance was carried out using F test. If necessary, Box-Cox transformation was used to meet the requirement of normality and equal variances before comparing means.

**References to Supplemental Methods:**


### Supplemental Table 1. Sequences of primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nppa</td>
<td>5´-CTCCCAGGCCATATTGGAG-3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-TCCAGGTTGCTAGCAGGTT-3´ (reverse)</td>
</tr>
<tr>
<td>Nppb</td>
<td>5´-TGGGAAGTCTAGCCAGTCTC-3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-TCTGAGCCATTTCTCTGAC-3´ (reverse)</td>
</tr>
<tr>
<td>Actb</td>
<td>5´-CTCTGAACCCTAAGGCAAC-3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-ACCAGAGGCATAAGGACA-3´ (reverse)</td>
</tr>
<tr>
<td>Mapk1</td>
<td>5´-TCTCCCGCAACAAAAATAAGG-3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-GCCAGAGCCTGTCAACTTC-3´ (reverse)</td>
</tr>
<tr>
<td>Igf1r</td>
<td>5´-AGCCATGGTGAGAACAC-3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-CGCACACGCTTTTGAGTAG-3´ (reverse)</td>
</tr>
<tr>
<td>Grb2</td>
<td>5´-GGGGATTTTCTCCCTGTCAGT-3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-CTGTCTATGTCCCAGAGAA-3´ (reverse)</td>
</tr>
<tr>
<td>Ksr1</td>
<td>5´-CCAAGGCCCTAACAAAGAG-3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-GGAAGTTGACACTGCTCC-3´ (reverse)</td>
</tr>
<tr>
<td>Myh7</td>
<td>5´-GGATGACGTTACCTCAACA -3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-GTGTTCCTGACCTTGCTC-3´ (reverse)</td>
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<tr>
<td>Med13</td>
<td>5´-TTTACCACCTCAACTTCCA -3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-AAGGCTTTTACACTGCAAC -3´ (reverse)</td>
</tr>
<tr>
<td>Crat</td>
<td>5´-AACGCTACAGAAGGACTG -3´ (forward)</td>
</tr>
<tr>
<td></td>
<td>5´-GGGCTGAGTAGATGACCAC -3´ (reverse)</td>
</tr>
</tbody>
</table>
Fig. S1. Determination of cardiomyocyte apoptosis.

(A) miR-378 increases apoptosis in NRCM. Apoptosis was analysed in isolated NRCM by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Representative pictures of TUNEL staining, 96 hours after transfection with synthetic miR-378 or a scrambled sequence (miR-ctrl). The quantification shows results from 3 independent experiments performed in duplicate. Arrows indicate apoptotic cell nuclei, scale bar = 50 μm.

(B) Inhibition of endogenous miR-378 reduces apoptosis in NRCM. Experiments as in (A), except that antimiR-378 or antimiR-ctrl were transfected. Results are from three independent experiments performed in duplicate.

(C) Exogenous expression of miR-378 does not affect cardiomyocyte apoptosis in vivo. Apoptosis was analysed in paraffin sections of mouse hearts by TUNEL staining. Representative pictures of TUNEL staining in ctrl- and AAV-miR-378-treated mouse hearts. (Right) Quantification of apoptosis in hearts from mice that had been infected with AAV-miR-378 after TAC (n=7 animals) or sham surgery (n=5). Data from non-infected sham- or TAC-treated control mice are shown in parallel (n=5 each with four fields of view per animal), scale bar = 100 μm.
**Fig. S2. Myocardial expression of miR-378 during development and after birth.** Levels of miR-378 were determined by quantitative real-time PCR in RNA prepared from myocardial tissue obtained at the age indicated. (n=1 for E15.5, E16.5, E17.5, E18.5; n=4 for D1, D2, D30, D120).
### Supplementary Figure S3

**Grb2**
- Native: 5’ UAAAGAUAGUCCAGAGCUCUUUC 3’
- Mutated: 5’ UAAAGAUACGUCAGCUCUUUC 3’

**Igf1r**
- Native: 5’ GCUCUUAAGUCCAGUAGAUUAC 3’
- Mutated: 5’ GCUCUUAACGUCAGUAGAUUAC 3’

**Ksr1**
- Native: 5’ GUCCUGGAAGUCCAGAUUAC 3’
- Mutated: 5’ GUCCUGGAACGUAGAUUAC 3’

**Mapk1**
- Native: 5’ AUUCUUCAGUCCAGAGUUCT 3’
- Mutated: 5’ AUUCUUCACGUCAGAGUUCT 3’

**Red = seed sequence**
**Green = mutated sites**

**Fig S3.** Native miR binding sites in the 3’UTR of Grb2, Igf1r, Ksr1 and Mapk1 and their mutation for luciferase reporter assays. Nucleotides that are complementary to the seed sequence of miR-378 are shown in red and the mutated bases are shown in green.
**Fig S4. Knockdown efficiency of siRNAs against Grb2, Igf1r, Ksr1 and Mapk1.** NRCM were transfected with 100nM si-Grb2, si-Igf1r, si-Ksr1 and si-Mapk1 24 hours after isolation. RNA was isolated 48 hours after transfection and quantitative PCR was performed to determine the respective mRNA levels. The quantification shows results from 2-3 independent experiments. Student's t test was used for statistical analysis.
**Fig. S5.** Assessment of parameters for cardiac morphology and function indicates no difference between AAV9-GFP and PBS-control. To test whether an intrinsic property of AAV9 causes the beneficial effects observed with AAV9-miR-378, a control construct for expression of GFP from an AAV9 vector was created (AAV9-GFP) and compared to a PBS control. The same procedure as used in the experiments of Fig. 4 was applied. In brief, 5 weeks-old wildtype male mice were treated by tail vein injection with either AAV9-GFP (1 x 10^{12} genome copies/mouse) or PBS. After 3 weeks, thoracic aortic constriction (TAC) was performed. Echocardiographic analysis was performed at 11 weeks of age. Shown here are the LV weight/tibia length data for the two groups along with echocardiography data for fractional shortening, ejection fraction, LV internal diameter (systole and diastole) and LV posterior wall thickness (systole and diastole). Group sizes are: 12 and 4 sham-treated mice that received AAV9-GFP or PBS, respectively and 7 and 6 TAC-treated mice that received AAV9-GFP or PBS, respectively.
Fig. S6. AAV-miR-378 reduces the expression of hypertrophic marker genes, yet leaves miR-1 and miR-133a as well as miR-378-5p and its targets unaffected.

(A) Quantitative PCR analysis to determine the mRNA level of hypertrophic markers atrial natriuretic peptide (Nppa), brain natriuretic peptide (Nppb) and beta-myosin heavy chain (Myh7) was performed in myocardial tissue obtained from AAV9-miR-378-treated mice and control mice after sham or TAC treatment. Group sizes are: 5 and 11 sham-treated mice that received AAV9-miR-378 or PBS, respectively and 7 and 8 TAC-treated mice that received AAV9-miR-378 or PBS, respectively.

(B) Myocardial expression of miR-378-5p (miR-378*), miR-1 and miR-133a. Levels of miR-378, miR-378*, miR-1 and miR-133a were determined by quantitative real-time PCR in RNA prepared from myocardial tissue obtained from AAV-miR-378-treated mice and control mice. The quantification for miR-378* shows results from 5 and 10 sham-treated mice that received AAV9-miR-378 or PBS respectively, and 7 and 8 TAC-treated mice that received AAV9-miR-378 or PBS, respectively. Levels of miR-1 and miR-133a were determined from 5 sham- treated mice that received AAV9-miR-378 or PBS and 7 and 5 TAC-treated mice that received AAV9-miR-378 or PBS, respectively. Data for miR-378-3p are from Fig. 4B and are marked in grey.

(C) Quantitative PCR analysis of Med13 and Crat in myocardial tissue obtained from AAV9-miR-378-treated mice and control mice after sham or TAC treatment. Group sizes are: 5 sham-treated mice that received AAV9-miR-378 or PBS, and 7 and 5 TAC-treated mice that received AAV9-miR-378 or PBS, respectively.