A Cardiac-enriched MicroRNA, miR-378, Blocks Cardiac Hypertrophy by Targeting Ras Signaling

Received for publication, December 4, 2012, and in revised form, January 29, 2013  Published, JBC Papers in Press, February 27, 2013, DOI 10.1074/jbc.M112.442384

Raghu S. Nagalingam, Nagalingam R. Sundaresan, Mahesh P. Gupta, David L. Geenen, R. John Solaro, and Madhu Gupta

From the Department of Physiology and Biophysics and Center for Cardiovascular Research, University of Illinois, Chicago, Illinois 60612 and the Department of Surgery, Committee on Cellular and Molecular Physiology, University of Chicago, Chicago, Illinois 60637

Background: miR-378 is a newly discovered cardiomyocyte-enriched miRNA.

Results: By targeting Grb2, miR-378 blocks activation of the hypertrophic signaling cascade and gene expression. Its deficiency contributes to the development of hypertrophy in a Ras activity-dependent manner.

Conclusion: miR-378 is a negative regulator of cardiac hypertrophy.

Significance: Cellular restoration of miR-378 will be beneficial in preventing adverse cardiac remodeling.

Understanding the regulation of cardiomyocyte growth is crucial for the management of adverse ventricular remodeling and development of hypertrophy. Cardiac hypertrophy is an adaptive response of myocytes to increased workload that often develops as a consequence of heart failure.

This article has been withdrawn by the authors. Lanes 1 and 2 of the actin immunoblot in Fig. 3B were reused in lanes 3 and 4 of the actin immunoblot in Fig. 7C. The ANF immunoblot in Fig. 3B was reused as ANF in Fig. 9F. Control lanes 1 and 2 of the ANF immunoblot in Fig. 8C were duplicated. The Grb2 and GAPDH immunoblots in Fig. 9C were inappropriately manipulated.

These changes are initially considered as compensatory responses of the heart that prevent failure. However, under certain conditions, this adaptive process may become deleterious, leading to heart failure.

In studies reported here we demonstrate that the microRNA, miR-378, acts as an endogenous negative regulator of Ras signaling and development of cardiac hypertrophy. Ras belongs to a family of small G-proteins, which act as molecular switches by alternating between the GTP-bound active form and the GDP-bound inactive form. Activation of Ras requires recruitment of adapter protein Grb2 (growth factor receptor bound) to the membrane and its interaction with the SOS (son of sevenless). Ras signaling has been shown to play a critical role in the regulation of the hypertrophic growth process of the heart.

It serves as a nodal point for transmitting signals originating from multiple growth factor receptors and their ligands such as receptor tyrosine kinases (IGF2 receptor, epidermal growth factor receptor, and platelet-derived growth factor receptor) and agonists (IGF-1, epidermal growth factor, and platelet-derived growth factor), G-protein-coupled receptors, and cytokines.

The abbreviations used are: IGF-1, insulin-like growth factor-1; GSK-3β, glycogen synthase kinase-3β; NFAT, nuclear factor of activated T cells; mTOR, mammalian target of rapamycin; PE, phenylephrine; BNP, brain natriuretic peptide; ANF, atrial natriuretic factor; Ang II, angiotensin II; HCM, hypertrophic cardiomyopathy.

* This work was supported, in whole or in part, by National Institutes of Health Grants Multi P.I.: RO1 HL 22231 (to M. G. and R. J. S.), RO1 HL 83423 (to M. P. G.), and PO1 HL 062426 (to R. J. S.).

† This article contains supplemental Figs. S1–S6 and Table S1.

‡ To whom correspondence should be addressed: Dept. of Physiology and Biophysics (MC 901), University of Illinois, 835 S. Wolcott Ave., Chicago, IL 60612. Tel.: 312-355-4937; Fax: 312-969-1414; E-mail: guptam@uic.edu.
microRNAs are small noncoding RNAs that have emerged as powerful endogenous negative regulators of gene expression. Among more than 1000 miRNAs described so far in humans, relatively few (example, miR-1, miR-208, miR-499, and miR-133) show high levels of expression in the heart. The gain- and loss-of-function studies have shown important regulatory roles of cardiac-enriched miRNAs in the process of cardiac development (miR-1 and miR-133) and in the settings of cardiac diseases such as arrhythmias (miR-1, miR-133, and miR-208a), myocardial infarction (miR-499), and pressure overload-induced cardiac hypertrophy (miR-208a, miR-1, and miR-133) (reviewed in Ref. 12). Recently, some of these miRNAs have also emerged as biomarkers against postnatal cardiac remodeling and heart failure, thus further highlighting the importance in the diagnosis and treatment of cardiac diseases (reviewed in Ref. 13). We have recently identified a new member of cardiac-enriched miRNAs (miR-378) as a potential cardiac hypertrophy modulator and an essential component of the Ras signaling pathway, as a direct target of miR-378.

miR-378 blocks hypertrophy agonist-stimulated Ras activation and downstream effectors of the PI3K/AKT and Raf-MEK1 signaling pathways thereby promoting anti-growth activity of GSK-3β and inhibiting transcriptional activity of NFAT and ERK. The cumulative effect leads to suppression of fetal gene expression and inhibition of hypertrophy of cardiomyocytes. We also show that the stimuli, which promote development of cardiac hypertrophy, cause down-regulation of miR-378. These data suggest that inhibition of miR-378 by hypertrophy agonists contributes to the development of cardiac hypertrophy. Therefore, replenishing miR-378 levels may be beneficial to protect the heart developing pathological remodeling and descending to heart failure.
Induction of Hypertrophy

More than 80% using sense strand as a negative control (16).

RNA Interference

For endogenous inhibition of Grb2 expression, we used a duplex siRNA corresponding to human Grb2 coding nucleotides 607–627 (Thermo Scientific). This siRNA was previously shown to effectively inhibit Grb2 expression in HeLa cells by more than 80% using sense strand as a negative control (16).

Induction of Hypertrophy

Cell Culture—Cardiomyocytes were exposed to 20 μM phenylephrine (PE) or 100 nM angiotensin II (Ang II) or 10 μM isoproterenol for 48 h in serum-free defined medium (Opti-MEM; Invitrogen).

Animals—All animal protocols were reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee. Isoproterenol (ISP, 8.7 mg/kg/d) was delivered chronically for 7 days by implanting osmotic mini pumps (ALZET model 2001, flow rate 1 μl/h/day) in the peritoneal cavity of mice. Control mice underwent the same procedure except that their pumps were filled with vehicle. Pressure overload hypertrophy was induced by transverse aortic constriction of ascending aorta of mice, as described elsewhere (17). Volume overload was induced in adult Sprague-Dawley rats by aorto-caval shunt as per our published protocol (18). The study was approved by the University of Illinois Institutional Animal Care and Use Committee. Isoproterenol (ISP, 8.7 mg/kg/d) was delivered chronically for 7 days by implanting osmotic mini pumps (ALZET model 2001, flow rate 1 μl/h/day) in the peritoneal cavity of mice. Control mice underwent the same procedure except that their pumps were filled with vehicle. Pressure overload hypertrophy was induced by transverse aortic constriction of ascending aorta of mice, as described elsewhere (17). Volume overload was induced in adult Sprague-Dawley rats by aorto-caval shunt as per our published protocol (18).

Patient Samples—Cardiomyocytes were harvested from the Tissue Repository Bank maintained at the Loyola University Medical Center. The study was approved by the Institutional Review Board of Loyola University Medical Center and the University of Illinois, Chicago, and performed in accordance with NIH guidelines.

Northern Analysis

Total RNA was isolated resolved in 12% urea-PAGE, transferred, and processed for hybridization with radiolabeled probes as per our published protocols (14). After washing, the signal was captured by exposing the membranes to the phosphorimaging screen followed by scanning on a Storm 860 scanner. Signal intensity was quantified using the ImageJ program. The U6-labeled radioactive probe was used as loading control for all samples after stripping the membranes.

Immunoprecipitation and Western Analyses

Western blotting and immunoprecipitation experiments were done using a standard protocol as described elsewhere (20).

[3H]Leucine Incorporation Assay

Immediately after treatment of cardiomyocytes with PE, cells were incubated with [3H]leucine (1.0 mCi/ml, 163 Ci/mmol specific activity, Amersham Biosciences) in leucine-free minimal essential medium for 48 h. Cells were washed and processed for leucine incorporation as described previously (21). Values were normalized with DNA content, measured by the Quant-iT picogreen double-stranded DNA assay kit (Invitrogen).

Immunostaining of Cells

Cardiomyocytes (10,000 to 20,000) were plated on 1% fibronectin-coated coverslips and processed for immunostaining as per the protocols described previously (14). Sarcomere reorganization and ANF release were measured by staining the cardiomyocytes with antibodies specific for α-actinin and ANF, respectively. For analysis of cellular distribution of NFAT, cells were immunostained with protein-specific antibodies, and the protein was localized by use of a confocal microscopy. Cell imaging was performed on a Bio-Rad Laser Sharp 2000 system (Bio-Rad) using a ×40 objective (Zeiss). For each experimental group, there was a minimum of three experiments with at least three replicates of each sample.

Dual Luciferase Reporter Assay

Firefly luciferase activities were measured sequentially using the Dual Luciferase Reporter Assay System as described previously (22). The firefly luciferase signal was measured, followed by the Renilla luciferase signal using a EG&G Berthold LUMAT luminometer. Firefly luciferase activity was normalized using Renilla luciferase signal and values were expressed as arbitrary light units.

Real-time Polymerase Chain Reaction

Real-time PCR primers for mouse ANF, Grb-2, and β-MHC were designed using Primer Design software, the sequence is available upon request. Total RNA was isolated, treated with DNase, and reverse transcribed using the SuperScript III kit and random hexamers. In a 20-μl PCR, 5 ng of cDNA template was mixed with primers to a final concentration of 200 nM and 10 μl of Fast SYBR Green master mix. Amplification was carried out in a 7500 Fast Real-time PCR system by first incubating the reaction mixture at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C for 30 s. For quality control purposes, at the end of each run, dissociation curves were generated by incubating the reactions at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. Primer pairs used in the study were free of primer dimer artifacts. Expression ratios were calculated by the ΔΔCt method, where Ct is the cycle threshold, using GAPDH as a reference gene.

Statistical Analysis

Data are expressed as the mean ± S.D. of at least 3 independent experiments. Control and treatment groups were matched in sets containing cells isolated and cultured on the same day to eliminate variability due to a cell batch. The two-tailed Student’s t test was used for performing analysis of variance in Excel. A p value of 0.05 or less was considered statistically significant.
RESULTS

Cardiac Expression of miR-378 Is Repressed during Development of Cardiac Hypertrophy—To investigate whether miR-378 is involved in the development of cardiac hypertrophy we measured miR-378 levels in cardiomyocytes treated with hypertrophic agonists (PE, Ang II, and isoproterenol) for 48 h. Hypertrophy of cardiomyocytes was confirmed by measuring [3H]leucine incorporation into total proteins and cell size. The results showed that all three agonists caused an approximately 150 to 200% increase in cell size and about 100 to 140% increase in [3H]leucine incorporation. This was associated with a significant reduction in the expression level of miR-378 (Figs. 1, A and B). We next examined miR-378 expression levels in three different in vivo models of cardiac hypertrophy: pressure overload by creating transverse aortic constriction in adult mice for 4 weeks, volume overload by inducing aorto-caval shunt in adult rats for 6 weeks, and isoproterenol infusion by implanting mini osmotic pumps in adult mice for 2 weeks. As reported in our previous publications, all three interventions produced a significant amount of cardiac hypertrophy (21–23). In these in vivo models, expression levels of miR-378 was reduced by 40 to 60% compared with sham controls (Fig. 1, C-E). We also measured miR-378 levels in patients with end-stage heart failure diagnosed with nonischemic dilated cardiomyopathy and compared it with nonfailing hearts. We found that out of three patients that were analyzed, two showed a significant reduction in miR-378 expression. These findings thus indicate that reduced expression of miR-378 is associated with the development of cardiac hypertrophy in animal models as well as in select cases of human heart failure.

Overexpression of miR-378 Blocks Cardiac Hypertrophy—To test whether replenishing miR-378 levels during hypertrophy could block hypertrophy of cardiomyocytes, we overexpressed miR-378 by using a double-stranded synthetic miR-378-mimic (miR-378), a control oligo (mimic-C) designed with no homology to any known miRNA sequences in the database served as negative control. Primary cultures of cardiomyocytes were transfected with increasing amounts (10–50 nM) of miR-378, which led to a dose-dependent increase in the expression levels of miR-378. Subsequent experiments were performed with a 25 nM dose of miR-378, which produced nearly 5–10-fold higher expression of mature miR-378 relative to the endogenous levels (Fig. 2A). Cardiomyocytes overexpressed with miRNA were then treated with PE (20μM) or Ang II (100 nM, twice 24 h apart) for 48 h, and their hypertrophic response was measured by monitoring induction of protein synthesis. We found that miR-378 overexpression significantly reduced PE- and Ang II-induced protein accumulation, as measured by [3H]leucine incor-
MicroRNA-378 Targets Ras Signaling in Cardiac Myocytes

poration into total cellular proteins (Fig. 2B). We also monitored the influence of miR-378 gain-in-function on PE-stimulated changes in cell morphology and size. By confocal imaging we found that PE treatment for 48 h caused more regular cell shape of cardiomyocytes with myofibrils organized into sarcomeres. These cells showed a significant increase in cell surface area. Increased expression of miR-378 by itself had no significant effect on cell morphology or cell size, it significantly interfered with PE-stimulated myofibrillar organization to the extent that miR-378 overexpressing cells had a more regular shape, their myofibrillar organization appeared similar to untreated rather than PE-stimulated control cells (Fig. 2C).

Overexpression of miR-378 significantly reduced the PE-stimulated increase in cell size (Fig. 2D). Induction of fetal gene expression is considered a hallmark of cardiac hypertrophy. We therefore measured the effect of miR-378 on the PE-stimulated induction of ANF, skeletal actin, and brain natriuretic peptide (BNP) expression in cardiomyocytes. Overexpression of miR-378 by itself had no significant effect on ANF release or expression as evaluated by confocal imaging, by Western blotting or real-time PCR (Fig. 3A–C). Upon PE stimulation, high perinuclear ANF staining, which was noted in cardiomyocytes transfected with control mimic-C, was found significantly reduced in cells overexpressed with miR-378 (Fig. 3A). This was confirmed quantitatively by Western blot and real-time PCR analyses, which showed a significant reduction in ANF protein levels and mRNA levels (Fig. 3B and C). There was also a significant reduction in the mRNA levels of skeletal actin and BNP by miR-378 upon PE treatment (Fig. 4A). We also examined the promoter activity of β-MHC, which is a well known marker of the fetal gene program and pathologic cardiac remodeling (3). By transient transfection assays we found that overexpression of miR-378 significantly reduced the PE-stimulated induction of β-MHC-luciferase activity in cardiomyocytes (Fig. 4B).

We next analyzed nuclear accumulation of NFAT, another marker for induction of the hypertrophic gene program. Under basal conditions the NFAT proteins are phosphorylated and reside in the cytoplasm. But upon stimulation of cells by hypertrophic agonists they are dephosphorylated by calcineurin, resulting in translocation to the nucleus, where they cooperate with other muscle transcription factors and activate the hypertrophic gene program (24). We used confocal microscopy to examine subcellular distribution of NFAT in cardiomyocytes...
MicroRNA-378 Targets Ras Signaling in Cardiac Myocytes

transfected with miR-378 or mimic-C control. Under basal nonstimulated conditions, miR-378 had no noticeable effect on NFAT subcellular distribution. Following PE treatment for 48 h, cells transfected with mimic-C had a considerable amount of NFAT localized in the nucleus, whereas NFAT staining was minimal in the nuclei of cells overexpressing miR-378. We next examined whether miR-378 has the ability to regulate protein synthesis. For this purpose we measured p70S6 kinase activity, a key signaling kinase regulating translation of protein synthesis (25, 26). The results showed that PE stimulation of cardiomyocytes enhanced the phosphorylation of p70S6 kinase in mimic-C-transfected cells, which was found considerably reduced by overexpression of miR-378 (Fig. 5A, top panel). Together, these results demonstrated that miR-378 has the ability to inhibit agonist-stimulated hypertrophy of cardiomyocytes.

miR-378 Inhibits Activation of MAPK/ERK and PI3K/AKT Signaling Pathways—Having known that both transcription and translation events inducing cardiomyocyte hypertrophy were significantly inhibited by miR-378; we next searched for signaling mechanisms that contributed to this effect. In the case of activation of G protein-coupled receptors by agonists such as PE, isoproterenol, or Ang II, sequential activation of MAPK, PI3K, and AKT has been documented (4). Among the MAPKs, ERK1/2 is considered to be a central regulator of agonist-mediated cardiac hypertrophy (4). We therefore examined the effect of miR-378 on the activation of ERK1/2 at different time points (5 and 15 min) after stimulation of cells with PE. The results showed that PE induced the phosphorylation of ERK1/2 in cells overexpressing mimic-C, which was found significantly reduced in cells overexpressed with miR-378 (supplemental Fig. S1). Some previous reports have shown that prolonged exposure of cells to PE is required to detect alterations in gene expression and protein synthesis, and that the initial peak of ERK1/2 activity is not sufficient to trigger the hypertrophic response (27). To address this issue, we studied phosphorylation of ERK1/2 in miR-378 overexpressing cells after 48 h of PE treatment. We again found that miR-378 significantly inhibited PE-mediated activation of ERK1/2 as compared with the mimic-C control (Fig. 5, A and B), suggesting that miR-378 is capable of suppressing the MAPK/ERK1/2 signaling pathway activity. We then examined the role of miR-378 in regulating activity of the PI3K/AKT pathway. AKT is activated by various extracellular stimuli in a PI3K-dependent manner by an upstream kinase PIKK. The downstream targets of AKT include GSK-3β, mTOR, and mTOR. During cardiac hypertrophy GSK-3β activity is shown to be phosphorylated by AKT, leading to suppression of its kinase activity (28). By analyzing phosphorylation of these proteins, we found that PE stimulation induced phosphorylation of AKT and GSK-3β, but not by overexpression of miR-378, but not by overexpression of miR-378 (Fig. 5C). Additionally, we observed increased expression of ANF upon PE treatment as measured by Western analysis of ANF protein, and that this induction was reduced by overexpression of miR-378 upon PE treatment as measured by real-time PCR analysis of ANF mRNA expression (Fig. 6A). These results thus demonstrated that overexpression of miR-378 reduces the activity of pro-hypertrophic MEK/ERK1/2 and PI3K-AKT signaling, and enhances the activity of anti-hypertrophic GSK-3β signaling pathways.

Inhibition of Endogenous of miR-378 Increases Cardiomyocyte Cell Size, and Potentiates PE-mediated Hypertrophic Signaling and Activation of Fetal Gene Program—We next took a complementary approach to address whether inhibition of endogenous miR-378 would induce the hypertrophic response. For inhibition of miR-378, we used a commercially available miR-378 anti-miR, and a single-stranded scramble control (scramble-C), designed not to target any known miR sequences, served as a negative control. Transfection of cardiomyocytes with anti-miR resulted in complete elimination of endogenous miR-378 expression (Fig. 6A). Inhibition of miR-378 alone was found sufficient to induce an almost 30% increase in cardiomyocyte size (Fig. 6, B and C) and it induced the mRNA levels of fetal genes, BNP by 5-fold and skeletal actin by 2.5-fold (Fig. 6D). There was also an increase in ANF release around 5-fold in ANF protein expression (Fig. 7, A and B). These results indicated that basal activity of miR-378 must be essential for restraining fetal gene expression in nonstimulated conditions.
We next examined the effect of miR-378 inhibition on PE-stimulated changes in cell morphology, cell size, and on fetal gene expression. The majority of PE-treated control cells showed a well-defined shape with organized myofibrils, anti-miR transfected cells, although showed similarly organized myofibrils that appeared more irregular in shape, were about 40% larger than PE-stimulated control cells (Fig. 6, B and C).

There was also a higher induction in mRNA levels of skeletal actin (50%) and BNP (30%) in anti-miR-transfected cells as compared with control cells (Fig. 6, D). This was associated with intense perinuclear release of ANF compared with PE-treated controls (Fig. 7, A). The results obtained from Western blotting also confirmed nearly 2.5-fold higher induction of ANF by PE treatment in the anti-miR group, compared with control cells (Fig. 7, B). We next analyzed activation of p-p70S6K and pERK1/2 and found no changes in nonstimulated cells with anti-miR. Upon PE stimulation, a significantly higher phosphorylation of both signaling kinases was observed in the anti-miR group, as compared with control cells (Fig. 7, C and D), suggesting higher activation of the hypertrophy inducing signaling cascade by miR-378 inhibition.

**miR-378 Is a Negative Regulator of Ras Signaling**—Because the activity of both the ERK1/2 and AKT signaling pathways was suppressed by miR-378, we hypothesized that there might be a common upstream target that regulates the activity of both of these pathways. We focused on Ras, a small (21 kDa) GTP-binding protein, which plays a pivotal role in the development of cardiac hypertrophy, and which is capable of regulating the activity of both ERK1/2 and AKT pathways (29, 30). Ras is biologically active when bound to GTP and becomes inactive as a result of its innate GTPase activity, which hydrolyzes the bound GTP to GDP (5).

---

**FIGURE 4. miR-378 inhibits cardiac hypertrophy response.** A, real-time PCR analysis of mRNA levels of skeletal actin (skel actin) and BNP normalized with GAPDH. Cells were transfected and treated with PE as described in the legend to Fig. 2C. B, luciferase activity in cardiomyocytes transfected with the β-MHC promoter/luciferase reporter construct along with miR-378 or mimic-C, 24 h later, cells were treated with PE for 48 h. Each bar represents mean ± S.D. of triplicates of three independent experiments. Dagger, significant from vehicle-treated control; asterisk, significant (p < 0.05) when compared with PE-treated control. C, confocal imaging of NFAT immunostaining (green) in cardiomyocytes transfected with mimic-C or miR-378, after 24 h, cells were treated with PE for 48 h. To-pro stain (blue) marks nuclei positions.
miR-378, we performed a Ras activity assay. In this assay, active Ras is co-precipitated with the Ras-binding domain of Raf (Raf-RBD), which specifically binds to Ras-GTP (active Ras). As shown in Fig. 8, A and B, higher levels of active Ras was observed in PE-treated cardiomyocytes transfected with control mimic-C, compared with cells overexpressed with miR-378. This data suggest that miR-378 has the potential to block the agonist-mediated activation of Ras. To biochemically titrate the effect of miR-378 on Ras signaling, we used a recombinant adenovirus Ad-rasN17 encoding the dominant-negative Ras protein. Ras-N17 is a Ras mutant protein with substitution of asparagine (Asn) in place of serine (Ser) at position 17. This mutant inhibits the function of all endogenous cellular Ras proteins and prevents phosphorylation of mitogen-activated protein kinases and cell proliferation of myoblasts upon serum stimulation (31). We validated the efficacy of the Ad-rasN17 mutant in cardiomyocytes by finding the reduced AKT phosphorylation after serum stimulation (supplemental Fig. S3). We next tested whether induction of ANF by miR-378 inhibition (as shown in Fig. 7B) is dependent on the activity of Ras. Cardiomyocytes were infected with Ad-rasN17 or with a blank adenovirus vector. The next day the same cells were overexpressed with anti-miR-378 or scramble-C under serum-free conditions and 24 h later they were switched to serum containing medium for an additional 24 h. As expected we found induced expression of ANF in anti-miR-378 expressing cells, which was significantly reduced in cells overexpressed with Ad-Ras-N17. In these experiments we found no alteration in ANF expression between cells expressing Ad-Ras-N17 and Ad-blank virus in combination with scramble-C (Fig. 8, C and D). From these data we deduced that Ras signaling is effectively targeted by miR-378, and that ANF induction by miR-378 inhibition could be a result from activation of Ras signaling.

**Grb2, an Essential Component of Ras Signaling, Is a Direct Target of miR-378**—Because overexpression of miR-378 effectively inhibited Ras signaling, we next sought to identify direct targets of miR-378 involved in Ras signaling activation. We used the most commonly used bioinformatics prediction tools (Target scan (32), Pic tar (33), Diana-microT (34), miRanda (35) and miR database (36)) to identify the target. We limited our
search to those targets that are commonly predicted at least by two programs, and also to those that showed species conservation among vertebrates. Our search revealed multiple members that are activators of Ras signaling as potential targets of miR-378. These include Grb2, ERK2, RIT1, KSR1, and RAS.GRP4 (37–43). Interestingly, our search did not identify even a single member that is known to inactivate the Ras signaling pathway.

A list of candidate targets showing location of the miR-378 “seed” sequence and their role in the Ras signaling pathway is summarized in Table 1. Among the list of potential targets of miR-378, we focused on Grb2 for experimental validation. The species conservation miR-378 seed sequence in the 3′/H11032 UTR of Grb2 is shown in Fig. 8E. We next performed functional assays where the seed sequence of miR-378 from the 3′/H11032 UTR of Grb2 was cloned downstream of a luciferase reporter in a dual reporter construct. The wild type and mutant constructs were transfected into H9C2 cells, which we showed previously to express very low levels of miR-378, compared with cardiomyocytes isolated from neonatal hearts (14). In this assay we found that overexpression of miR-378 significantly inhibited the luciferase activity of WT, but not of the mutant 3′ UTR of Grb2 (Fig. 9A), thus suggesting that the 3′ UTR of Grb2 is responsive to miR-378-mediated regulation. We then examined the effect of miR-378 on the endogenous level of the Grb2 protein by expressing increasing amounts of miR-378. A dose-dependent reduction in the expression levels of Grb2 was observed in miR-378 overexpressing cells. The reduction in Grb2 levels ranged from 20% to 5-fold with increasing concentrations of miR-378 (Fig. 9B, supplemental Fig. S4A), thus suggesting that Grb2 is a valid target of miR-378. In our computational analysis, ERK2 also emerged as a potential target and activated ERK2 is a widely accepted growth promoting effector of Ras signaling. We therefore also analyzed ERK2 expression in the same lysate preparation, and found that ERK2 levels remain unchanged even in cells expressing very high levels of this micro-RNA (Fig. 9B), consistent with results presented in Fig. 5A. These data

FIGURE 6. miR-378 deficiency enhances cardiomyocyte hypertrophy. A, Northern blot showing knockdown of endogenous miR-378 expression in cardiomyocytes by anti-miR-378 (antimiR) or control (Scr-C), 72 h after transfection, U6 used as a loading control. B, confocal images of cardiomyocytes for cardiac cell morphology by α-H11032-actinin staining (green) of scr-C and anti-miR-transfected cells, treated with PE for 48 h. To-Pro stain was used to mark the position of nuclei (blue), note the sarcomeric organization and increased cell size by PE, which was further increased by inhibition of miR-378 by anti-miR. C, quantification of cell size of cardiomyocytes in scr-C and anti-miR-transfected cells under basal nonstimulated conditions (−) and upon PE stimulation (+). Cell size was quantified and analyzed as described in the legend to Fig. 2C. D, real-time PCR analysis for mRNA expression of fetal genes, skeletal actin, and BNP, normalized to GAPDH. Each bar is a mean ± S.D. of replicates with a minimum of n = 4 in each group. Dagger, significant when compared with nontreated control; asterisk, significant when compared with PE-stimulated control.
suggested that Grb2 and not ERK2 is a valid target of miR-378. Because miRNAs are known to repress expression of the target proteins by repressing protein translation and/or by promoting mRNA degradation, we next examined the effect of miR-378 overexpression on Grb2 mRNA levels. For this experiment, we tested miR-378 at 25 nM dose because it significantly downregulated (5-fold compared with mimic C) the Grb2 protein levels. After 72 h of transfection of cardiomyocytes with miR-378, or mimic-C, the Grb2 mRNA levels were analyzed by real-time PCR. The results showed nearly 2-fold reduction in the mRNA levels of Grb-2 by miR-378 overexpression (supplemental Fig. S4B). These results demonstrated that miR-378 targets Grb2 by causing translation inhibition as well as promoting its mRNA degradation.

Several previous studies have shown that Grb2 is an upstream component of Ras signaling adapter proteins essential for stimulation of Ras upon the mechanical stretch of cells (37, 44–46). However, the role of Grb2 in PE-stimulated hypertrophy and in human heart failure has not been documented. We therefore examined the Grb2 protein levels in PE-stimulated cells and found almost 2–2.5-fold increased expression by PE treatment as compared with controls. The increase in Grb2 expression was not observed in PE-treated miR-378 overexpressing cells (Fig. 9C, supplemental Fig. S5A). We next probed human failing hearts for the expression levels of Grb2 and found that out of 3 heart failure patients examined, 2 showed induction in Grb2 ranging from 3- to 6-fold (Fig. 9D, supplemental Fig. S5B). To our excitement these 2 patients also showed reduced miR-378 levels (as shown in Fig. 1F). The patient that had unchanged miR-378 expression showed no induction of Grb2. These results thus demonstrate a strong reciprocal relationship between Grb2 and miR-378 expression levels during induction of hypertrophy by PE and in patients with heart failure.

To further establish a role of miR-378 in Grb2 suppression, we used a siRNA duplex oligo, which was previously designed against human Grb2 coding nucleotides 607–627 and was shown to inhibit Grb2 expression by 90% in HeLa cells (16). Upon sequence alignment, this region of Grb2 was found to be fully conserved in rat and also in mice. This siRNA duplex pro-
duced about 70% inhibition of Grb2 protein levels in rat cardiomyocytes (Fig. 9E). Functional connection between miR-378 and Grb2 was then examined by using ANF induction as readout of miR-378 inhibition. We found that ANF induction by anti-miR-378 could be prevented by inhibition of endogenous Grb2 (Fig. 9F, and supplemental Fig. S6A). There was also a significant reduction (20%) in cell size that was induced by miR-378 inhibition (not shown). We also examined the effect of Grb2 siRNA on PE-stimulated induction of fetal gene expression and found that Grb2 inhibition significantly reduced mRNA levels of ANF, BNP, and skeletal actin (supplemental Fig. S6B). These results collectively suggested that Grb2 participates in PE-stimulated cardiac hypertrophy and that it is a bona fide target of miR-378.

**DISCUSSION**

This study was designed to investigate the role of miR-378 in the development of cardiac hypertrophy. By different experimental approaches, we show that miR-378 is an endogenous negative regulator of cardiac hypertrophy. The expression level of miR-378 is down-regulated during development of hypertrophy and in heart failure and overexpression of miR-378 inhibits hypertrophic growth of cardiomyocytes by interfering with the nuclear accumulation of NFAT and induction of the fetal gene.
MicroRNA-378 Targets Ras Signaling in Cardiac Myocytes

The isoform miR-378a (also known as miR-378a-3p and miR-378h, and miR-378i) are located as stand alone at various genomic locations (summarized in supplemental Table S1). The remaining four isoforms (miR-378c, miR-378d-1, miR-378d-2, miR-378e, miR-378f, and miR-378g) is embedded within the introns of the protein coding genes, whereas that of miR-378d-2, miR-378e, miR-378f, and miR-378g) is embedded within the introns of the pre-miR region of six isoforms (miR-378a, miR-378b, miR-378d-2, miR-378e, miR-378f, and miR-378g) is embedded within the introns of the protein coding genes, whereas that of the remaining four isoforms (miR-378c, miR-378d-1, miR-378d-2, miR-378e, miR-378f, and miR-378g) are so far the only known endogenous repressors of Ras signaling. Olsen’s group recently reported that deletion of one single pre-miR locus was lethal, Grb2 (H11001/H11002) is reported to enhance Ras-ERK signaling in cardiac fibroblasts. The cardiac phenotype of miR-378 deficiency was however, not described in this study. In our study, when we used anti-miR-378 that was designed to inhibit all isoforms of miR-378 in cardiomyocytes, we observed an increased cell size of cardiomyocytes and dramatic up-regulation of fetal gene expression including ANF, BNP, and skeletal actin. This was consistent with one of our earlier studies where we observed reduced expression of miR-378 in the fetal heart (where fetal genes are highly expressed) and robust expression of miR-378 in the adult heart (where fetal gene expression is minimal) (14). From these findings we deduce that cardiac abundance of miR-378 in adults plays an important role in maintaining cardiac cell homeostasis by keeping cardiac cell growth and the fetal gene program in check.

Several recent studies suggest that miRNAs serves as powerful endogenous regulators of cardiac growth, some promote hypertrophy (pro-hypertrophic miRNA), whereas others inhibit hypertrophy (anti-hypertrophic miRNA). During hypertrophy, miRNAs modulate the function of both muscle and nonmuscle cells. In fibroblasts, miR-21 promotes fibroblast differentiation and its expression is induced by hypertrophy agonists (47), whereas miR-29 and miR-30c inhibit fibrosis, their expression levels decline in response to hypertrophic stimuli (48, 51–53). In cardiomyocytes, the miRNAs that are known to promote hypertrophy include miR-208, miR-199a, miR-199b, miR-23a, miR-195, miR-100, and mir-18b and more.
recently the miR-212/132 family. All of these miRNAs, except miR-208a, show increased expression in response to hypertrophic agonists. Overexpression of these miRNAs enhances hypertrophic growth and their inhibition confers resistance to cardiac stress (51, 54–61). Among the anti-hypertrophic miRNAs, miR-98/let-7 regulates cardiac hypertrophy indirectly via thioredoxin (62), the other three miRNAs (miR-9, miR-1, and miR-133) could directly inhibit response of cardiomyocytes to hypertrophic stimuli (53, 63–69). Expression of these three miRNAs is down-regulated by hypertrophy agonists and their overexpression diminished the hypertrophic response. Our data presented here show that miR-378 is a new member of anti-hypertrophic miRNAs. Similar to previously described anti-hypertrophic miRNAs, the expression level of miR-378 is down-regulated by hypertrophic stimuli, both in vitro and in vivo in three different models of cardiac hypertrophy as well as in patients with heart failure, and its overexpression blocks the hypertrophic response of cardiomyocytes.

The effect of anti-hypertrophic miRNAs is known to be mediated by targeting distinct sets of molecular pathways. miR-9 directly targets myocardin and interferes with the nuclear activity of NFAT (69). miR-133a inhibits hypertrophy by direct targeting of calcineurin (68), NFAT (66), and Cdc42 and RhoA (63), miR-1 targets twinfilin-1 (70), and calmodulin-mediated hypertrophic signaling (64, 71). Genetic deficiency of miR-133a results in extensive fibrosis, impaired cardiac function, and pathological remodeling (72). Even though miR-133a is a cardiomyocyte-specific miRNA, it should be noted that it could also influence cardiac fibroblast function and reduce fibrosis by targeting connective tissue growth factor (53). miR-1 and miR-133a are cardiomyocyte-enriched miRNAs, both inhibit cardiac hypertrophy and both target IGF1 signaling (63, 64)

**MicroRNA-378 Targets Ras Signaling in Cardiac Myocytes**

**FIGURE 9.** Grb2 is a direct target of miR-378 in H9C2 cells showing the normalized luciferase activity in cells transfected with control, wild type, or mutated 3'UTR of Grb2 3'UTR or empty vector (Vector-C). B, Western blot showing the effect of miR-378 overexpression in cardiomyocytes transfected with control (Mimic-C) or miR-378. The endogenous levels of Grb2 and ERK2, the two predicted targets of miR-378 by computational algorithms. After 72 h cell lysates were analyzed for endogenous expression levels of ERK2 and Grb2. β-Actin was used as loading control. C, Western blot showing increased expression of Grb2 in PE-stimulated cardiomyocytes. Cells were transfected with control (mimic-C) or miR-378 and then treated with PE for 10 min or 48 h. A significant induction in Grb2 was noted at both time points of PE treatment in controls but not in miR-378 overexpressing cells. GAPDH used as loading control. D, Western blot demonstrating Grb2 expression in human nonfailing hearts (NF1, NF2, and NF3) and in patients with nonischemic cardiomyopathy (DCM1, DCM2, and DCM3). A significant induction of Grb2 was noted in 2 (DCM2 and DCM3) heart failure patients. E, Western blot showing that siRNA duplexes of Grb2 (40 nm) when transfected twice in cardiomyocytes at 24-h intervals produces about 70–80% inhibition in the expression levels of endogenous Grb2. F, knockdown of Grb2 prevents ANF induction triggered from miR-378 inhibition. Cardiomyocytes were transfected with Grb2 siRNA duplex or negative control siRNA as described in E in association with anti-miR-378 or scramble control in serum-free medium, 24 h prior to harvesting, cells were switched to serum containing medium.
MicroRNA-378 Targets Ras Signaling in Cardiac Myocytes

Hypertrophy agonists
Pressure overload
Volume overload

miR-378
Ras activation

Grb2

pAKT

pERK1/2

GSK3β

NFAT

MEF2

GATA4

Induction of fetal (ANF) genes and hypertrophy program

73). Similar to miR-1 and miR-133, we recently described miR-378 as a cardiomyocyte-specific miRNA in the heart, which inhibits IGF1 signaling by direct targeting of IGF1R during postnatal cardiac remodeling (14).

In this study, we provide evidence that similar to miR-1 and miR-133, miR-378 is also an anti-hypertrophic miRNA, but its anti-hypertrophic effects are mediated by repressing Ras activity. Although several components of the Ras-signaling pathway emerged as potential targets of miR-378 from our in silico analysis, we could identify one component, Grb2, as a direct target of miR-378. Grb2 is an adapter protein essential for Ras activation. A direct role of Grb2 in cardiac hypertrophy was demonstrated in a study of genetic deletion of Grb2, whereas germ line deletion of Grb2 was found to be embryonic lethal, Grb2"/" mice were found protected from pressure overload-induced cardiac hypertrophy (38), demonstrating a direct pathological role of Grb2 in the regulation of cardiac hypertrophy. Our study demonstrates that Grb2 expression is induced in patients with heart failure and also upon PE stimulation in cardiomyocytes, and that inhibition of Grb2 by siRNA interferes with PE-stimulated induction of fetal gene expression. We have demonstrated here that miR-378 is a strong repressor of Grb2. It directly binds to the 3' UTR of Grb2 mRNA and causes both translation inhibition as well as degradation of its mRNA thus profoundly inhibiting Grb2 expression. Although from our target prediction analysis, ERK2 also emerged as a potential target of miR-378, the expression levels of ERK2 were not affected with miR-378 overexpression, thereby excluding ERK2 as a direct target of miR-378. It remains to be seen whether other components of Ras signaling that emerged by in silico analysis could indeed turn out to be authentic targets of miR-378.

Ras signaling is known to play a significant role in the induction of pathological remodeling. Multiple hypertrophy agonists activate the Ras-signaling pathway (4, 6, 7) and ectopic activation of Ras signaling induces pathological cardiac remodeling (8–11). Ras signaling is activated by growth factor receptors (insulin-like growth factor receptor, epidermal growth factor receptor, and platelet-derived growth factor receptor), by G protein-coupled receptors (Ang II receptors, and α and β adrenergic receptors), and by integrin-focal adhesion kinase (induced by mechanical stretch). The two major downstream effectors for the Ras-signaling pathway include activation of PI3K-AKT and Ras-ERK signaling pathways. Although both of these Ras effectors pathways have also been implicated in pathological growth of the heart, their elimination leads to the development of heart failure (8, 74–76). We have recently shown that heart failure resulting from SIRT6 inhibition is due to a massive induction of IGF-1R signaling, which is caused by ras-ERK signaling (14), a possible contribution of this pathway in the anti-hypertrophic effects of miR-378 cannot be excluded. Therefore it appears that miR-378 acts multiple targets to repress Ras activation in cardiomyocytes; two of these, which we have demonstrated in this study, include IGF1R (as reported by us previously) and Grb2.

Considering the wide implications of Ras-ERK activation in cardiac pathophysiology, it should be noted that this signaling pathway has significant application in the development of a specific hypertrophic cardiomyopathy (HCM) associated with a syndrome named “RASopathy,” which comprises a set of phenotypically related, yet distinct, human developmental defects (e.g. Noonan, Costello, Cardio-Facial-Cutaneous, and LEOPARD syndromes) (77, 78). Some, but not all, of these syndromes develop HCM (79). The underlying cause of HCM in this syndrome is a high occurrence rate of dominant gain-of-function germ-line mutations in genes encoding members of Ras-ERK pathway (KRAS, NRAS, Raf1, SOS1, and PTPN11) (80–82). Mutant mouse models with specific mutations in these genes also recapitulate many features of the RASopathy syndrome including cardiac hypertrophy, ventricular dysfunction, and aberrant expression of fetal genes (83, 84). Although the occurrence rate of HCM is variable with mutations of different genes, in almost all cases, HCM is preventable by either genetic ablation of ERK1/2 or by pharmacological inhibition of MEK (85–87).

In summary, our study has defined a new role for miR-378 demonstrating its ability to block cardiac hypertrophy by preventing Ras activation. These findings have profound implications not only for the management of heart failure, but also for...
MicroRNA-378 Targets Ras Signaling in Cardiac Myocytes

HCM accompanying hyperactivation of Ras signaling in human congenital defects associated with RASoPathy.

Acknowledgment—We thank Dr. Allen Samuel (Loyola University Chicago Medical Center) for providing patient samples from failing, and nonfailing hearts.

REFERENCES

1. Hennersdorf, M. G., and Strauer, B. E. (2001) Arterial hypertension and cardiac arrhythmias. J. Hypertens. 19, 167–177
2. Vakili, B. A., Okin, P. M., and Devereux, R. B. (2001) Prognostic implications of left ventricular hypertrophy. Am. Heart J. 141, 334–341
3. Frey, N., Katus, H. A., Olson, E. N., and Hill, J. A. (2004) Hypertrophy of the heart. A new therapeutic target? Circulation 109, 1580–1589
4. Heineke, J., and Molkentin, J. D. (2006) Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat. Rev. Mol. Cell Biol. 7, 589–600
5. Sugden, P. H., and Clerk, A. (2000) Activation of the small GTP-binding protein Ras in the heart by hypertrophic agonists. Trends Cardiovasc. Med. 10, 1–8
6. Dorn, G. W., 2nd, and Force, T. (2005) Protein kinase cascades in the regulation of cardiac hypertrophy. J. Clin. Invest. 115, 527–537
7. Malumbres, M., and Barbacid, M. (2003) RAS oncogenes. The first 30 years. Nat. Rev. Cancer 3, 459–466
8. Hunter, J. J., Tanaka, N., Rockman, H. A., Ross, J., Jr., and Chien, K. R. (1995) Ventricular expression of a MLC-2v-ras fusion gene induces cardiac hypertrophy and selective diastolic dysfunction in transgenic mice. J. Biol. Chem. 270, 23173–23178
9. Ho, P. D., Zechner, D. K., He, H., Dillmann, W. H., Glombotski, C. G., McDonough, P. M. (1998) The Raf-MEK-ERK cascade represents a common pathway for alternation of intracellular calcium by kinases and by G protein-coupled receptors in cardiac myocytes. J. Biol. Chem. 273, 21517–21527
10. Harris, I. S., Zhang, S., Treskov, I., Kovacs, A., Weinheimer, C., and Musch, G. L., Dalamagas, T., Giannopoulos, G., Goumas, G., Koukis, E., Kourtis, A. J. (2004) Raf-1 kinase is required for myocardial cell survival and recovery in response to pressure overload. Circ. Res. 95, 718–723
11. Mitchell, S., Ota, A., Foster, W. R., Horvath, S., and Wang, Y. (2006) Delineating v-Src downstream effector pathways in transformed myocytes. Circ. Res. 99, 1179–1192
12. Small, E. M., Frost, R. J., and Olson, E. N. (2001) MicroRNAs add a new dimension to cardiovascular disease. Circ. Res. 89, 1022–1032
13. Dimmeler, S., and Zeiher, A. M. (2001) Modulating microRNAs. Novel biomarkers for cardiovascular diseases? Eur Heart J 31, 2705–2707
14. Knezevic, I., Patel, A., Sundaresan, N. R., Gupta, M. P., Solaro, R. J., Nagalingam, R. S., and Gupta, M. (2012) A novel cardiomyocyte-enriched microRNA, miR-378, targets insulin-like growth factor 1 receptor. Implications in postnatal cardiac remodeling and cell survival. J. Biol. Chem. 287, 12913–12926
15. Gupta, M., Žák, R., Libermann, T. A., and Gupta, M. P. (1998) Tissue-specific expression of the cardiac α-myosin heavy chain gene is controlled by a downstream repressor element containing a palindrome of two ets-binding sites. Mol. Cell. Biol. 18, 7243–7258
16. Záhradník, N., Oliva, J. L., Martínez, N., Jorge, R., Ballester, A., Gutiérrez-Eisman, S., García-Vargas, S., and Rojas, J. M. (2006) GRB2 is a negative modulator of the intrinsic Ras-GEF activity of Hrs1. Mol. Biol. Cell 17, 3591–3597
17. Sundaresan, N. R., Pillai, V. B., Wolfgeher, D., Samant, S., Vasudevan, P., Parekh, V., Raghuraman, H., Cunningham, J. M., Gupta, M., and Gupta, M. P. (2011) The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. Sci. Signal. 4, ra46
18. Ocampo, C., Ingram, P., Ilbawi, M., Arcilla, R., and Gupta, M. (2003) Revisiting the surgical creation of volume load by aorto-caval shunt in rats. Mol. Cell. Biochem. 251, 139–143
19. Sundaresan, N. R., Vasudevan, P., Zhong, L., Kim, G., Samant, S., Parekh, V., Pillai, V. B., Ravindra, P. V., Gupta, M., Jeevanandam, V., Cunningham, J. M., Deng, C. X., Lombard, D. B., Mostoslavsky, R., and Gupta, M. P. (2012) The sirtuin SIRT6 blocks IGF-Akt signaling and development of cardiac hypertrophy by targeting c-Jun. Nat. Med. 18, 1643–1650
20. Gupta, M. P., Samant, S. A., Smith, S. H., and Shroff, S. G. (2008) HDAC4 and PCAF bind to cardiac sarcomeres and play a role in regulating myofilament contractile activity. J. Biol. Chem. 283, 10135–10146
21. Sundaresan, N. R., Gupta, M., Kim, G., Rajamohan, S. B., Isbatan, A., and Gupta, M. P. (2009) Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. J. Clin. Invest. 119, 2758–2771
22. Freire, G., Ocampo, C., Ilbawi, N., Griffin, A. J., and Gupta, M. (2007) Overt expression of AP-1 reduces a myosin heavy chain expression and contributes to heart failure from chronic volume overload. J. Mol. Cell. Cardiol. 43, 465–478
23. Pillai, V. B., Sundaresan, N. R., Kim, G., Gupta, M., Rajamohan, S. B., Pillai, J. B., Samant, S., Ravindra, P. V., Isbatan, A., and Gupta, M. P. (2010) Exogenous NAD blocks cardiac hypertrophic response via activation of the SIRT3-LKB1-AMP-activated kinase pathway. J. Biol. Chem. 285, 3133–3144
24. Hogan, P. G., Chen, L., Nardone, J., and Rao, A. (2003) Transcriptional regulation by calcium, calcinurin, and NFAT. Genes Dev. 17, 2205–2232
25. Gingras, A. C., Raught, B., and Sonenberg, N. (2001) Regulation of translation initiation by FRAP/mTOR. Genes Dev. 15, 807–826
26. Vakili, B. A., Okin, P. M., and Devereux, R. B. (2001) Prognostic implications of left ventricular hypertrophy. Am. Heart J. 141, 334–341
27. Putney, V. (2000) Phosphorylation of the cap-binding protein eIF4E by PKC mediates hypertrophy of cardiac myocytes. Biochem. J. 371, 1–12
28. Frey, N., Katus, H. A., Olson, E. N., and Hill, J. A. (2004) Hypertrophy of the heart. A point of integration in hypertrophic signaling? Br. J. Pharmacol. 143, 147–153
29. Vakili, B. A., Okin, P. M., and Devereux, R. B. (2001) Prognostic implications of left ventricular hypertrophy. Am. Heart J. 141, 334–341
30. Putney, V. (2000) Phosphorylation of the cap-binding protein eIF4E by PKC mediates hypertrophy of cardiac myocytes. Biochem. J. 371, 1–12
MicroRNA-378 Targets Ras Signaling in Cardiac Myocytes

J. and Shaw, A. S. (2002) Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. Mol. Cell. Biol. 22, 3035–3045

40. Lee, C. H., Della, N. G., Chew, C. E., and Zack, D. J. (1996) Rin, a neuron-specific and calmodulin-binding small G-protein, and Rit define a novel subfamily of Ras proteins. J. Neurosci. 16, 6784–6794

41. Stone, J. C. (2011) Regulation and function of the RasGRF family of Ras activators in blood cells. Genes Cancer 2, 320–334

42. Yang, Y., Li, L., Wong, G. W., Kriils, S. A., Madhusudhan, M. S., Ali, S., and Stevens, R. L. (2002) RasGRF4, a new mast cell-restricted Ras guanine nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Identification of defective variants of this signaling protein in asthma, mastocytosis, and mast cell leukemia patients and demonstration of the importance of RasGRF4 in mast cell development and function. J. Biol. Chem. 277, 25756–25774

43. Ebinu, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, J. D. (2008) Dysregulation of miR-199a-5p links cardiomyocyte and endothelial cell hypertrophy and conduction in mice. Circ. Res. 93, 140–147

44. DiMichele, L. A., Doherty, J. T., Rojas, M., Beggs, H. E., Reichardt, L. F., Mack, C. P., and Taylor, J. M. (2006) Myocyte-restricted focal adhesion kinase deletion attenuates pressure-overload-induced hypertrophy. Circ. Res. 99, 636–645

45. Thum, T., Gross, C., Fiedler, J., Fischer, T., Kissler, S., Bussen, M.,uppo, P., Just, S., Rottbauer, W., Frantz, S., Castoldi, M., Soppa, J., Kotelskiany, V., Rosenwald, A., Basson, M. A., Licht, J., Rouhanifard, S. H., Muckenthaler, M. U., Tuschi, T., Buersachs, J., and Engelhardt, S. (2008) MicroRNA-378 controls cardiac disease by stimulating MAP kinase. J. Biol. Chem. 456, 980–984

46. Sayed, D., Rane, S., Lypowy, J., Malhotra, A., Vanter, D., and Ambros, M. Sprouty2 and promotes cellular reprogramming. Genes Cancer 9, 655–665

47. Casci, T., Vinós, J., and Freeman, M. R. (2011) KRAS GTPase inhibitor of Ras signaling. Cell 96, 655–665

48. CARR, D., Calcana, D., and Sen, R. H. (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc. Natl. Acad. Sci. U.S.A. 105, 13027–13032

49. Cuinders, R. F., Tijman, T. J., Schoen, B., Leenders, J., Lentink, V., van der Made, I., Herias, V., van Leeuwen, R. E., Schellings, M. W., Barenbrug, P., Maessen, J. G., Heymans, S., Pinto, Y. M., and Creemers, E. E. (2009) miR-133 and miR-30 regulate connective tissue growth factor. Implications for a role of microRNAs in myocardial matrix remodeling. Circ. Res. 104, 170–178

50. Callis, T. E., Pandya, K., Seok, H. Y., Tang, R. H., Tatsuguchi, M., Huang, Z. P., Chen, J. F., Deng, Z., Gunn, B., Shumate, J., Willis, M. S., Selzman, C. H., and Wang, D. Z. (2009) MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. J. Clin. Invest. 119, 2772–2786

51. Haghikia, A., Missol-Kolka, E., Tsikas, D., Venturini, L., Brundiers, S., Castoldi, M., Muckenthaler, M. U., Eder, M., Stapel, B., Thum, T., Haghikia, A., Petrach-Pawarz, E., Drexlher, H., Hilfiker-Klein, D., and Scherr, M. (2011) Signal transducer and activator of transcription 3-mediated regulation of miR-199a-5p links cardiomyocyte and endothelial cell function in the heart. A key role for ubiquitin-conjugating enzymes. Eur. Heart J. 32, 1287–1297

52. Rane, S., He, M., Sayed, D., Yan, L., Vanter, D., and Abdellatif, M. (2010) An antagonism between the AKT and β-adrenergic signaling pathways mediated through their reciprocal effects on miR-199a-5p. Cell. Signal. 22, 1054–1062

53. da Costa Martins, P. A., Salic, K., Gladka, M. M., Armand, A. S., Leptidis, S., Cloozouzi, H., Hansen, A., Coenen-de Roo, C. J., Bierhuizen, M. F., van der Nagel, R., van Kuijk, J., de Weger, R., de Bruijn, A., Condorelli, G., Arbones, M. L., Eschenhagen, T., and De Windt, L. J. (2010) MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling. Nat. Cell. Biol. 12, 1220–1227

54. Lin, Z., Murtaza, I., Wang, K., Jiao, J., Gao, J., and Li, P. F. (2009) miR-23a functions downstream of NFATc3 to regulate cardiac hypertrophy. Proc. Natl. Acad. Sci. U.S.A. 106, 12103–12108

55. van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, I., Gerard, R. D., Richardson, J. A., and Olson, E. N. (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc. Natl. Acad. Sci. U.S.A. 103, 18255–18260

56. Sucharov, C., Bristow, M. R., and Port, J. D. (2008) miRNA expression in the failing human heart: functional correlates. J. Mol. Cell. Cardiol. 45, 185–192

57. Ucar, A., Gupta, S. K., Fiedler, J., Erikkii, E., Kardasinski, M., Batkai, S., Nagy, L., Sadowski, S. D., Bang, C., Holzmann, A., Remke, J., Caprio, M., Jentzsch, C., Ertler, C., Geisendorf, S., Glas, C., Hofmann, T. G., Nessling, M., Arrowsmith, M., Carrier, L., Napp, L. C., Bauersachs, J., Chow, J., and Thum, T. (2012) The miRNA-212/132 family regulates calcineurin and cardiomyocyte autophagy. Nat. Metab. 1, 13–36

58. Matkovich, S. J., Wang, W., Tu, Y., Eschenbacher, W. H., Dorn, L. E., Marshall, W. S., Hill, J. A., and Olson, E. N. (2008) Dysregulation of miR-133 and miR-30 regulates connective tissue growth factor. Implications for a role of microRNAs in myocardial matrix remodeling. Circ. Res. 103, 185–192

59. Carè, D., Calcana, D., Felicetti, F., Bonci, D., Addario, A., Gali, P., Bagg, K. M., Segalini, P., Gu, Y., Dalton, N. D., Elia, L., Latronico, M. V., Heydal, M., Autore, C., Russo, M. A., Creemers, E. E., Frustaci, A., Calciulli, D., and Condorelli, G. (2007) Dysregulation of microRNA-1 and insulin-like growth factor-1 transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. Circulation 120, 2377–2385

60. Li, Q., Lin, X., Yang, X., and Chang, J. (2010) NFTATC4 is negatively regulated in miR-133a-mediated cardiomyocyte hypertrophic repression. Am. J. Physiol. Heart Circ. Physiol. 298, H1340–1347

61. Matkovich, S. J., Wang, W., Tu, Y., Eschenbacher, W. H., Dorn, L. E., Condorelli, G., Diwan, A., Nerbom, J. M., and Dorn, G. W., 2nd (2011) MicroRNA-133 protects against myocardial fibrosis and modulates electrical repolarization without affecting hypertrophy in pressure-overloaded adult hearts. Circ. Res. 106, 166–175

62. Dong, D. L., Chen, C., Huo, R., Wang, N., Li, Z., Tu, Y. J., Hu, J. T., Chu, X., Huang, W., and Yang, B. F. (2010) Reciprocal repression between miR-133a and calcineurin regulates cardiac hypertrophy. J. Biol. Chem. 285, 3277–3285

63. Wang, K., Long, B., Zhou, J., and Li, P. F. (2010) miR-9 and NFTATC4 regulate myocardin in cardiac hypertrophy. J. Biol. Chem. 285, 11903–11912

64. Li, Q., Song, X. W., Zou, J., Wang, G. K., Kremneva, E., Li, X. Q., Zhu, N., Sun, T., Lappalainen, P., Yuan, W. J., Qin, Y. W., and Jing, Q. (2010) Attenuation of microRNA-1 derepresses the cytoskeleton regulatory protein twinfilin-1 to provoke cardiac hypertrophy. J. Cell Sci. 123, 2444–2452

65. Obata, K., Nagata, K., Iwase, M., Odashima, M., Nagasaka, T., Izawa, H., Murohara, T., Yamada, Y., and Yokota, M. (2005) Overexpression of cal-
modulin induces cardiac hypertrophy by a calcineurin-dependent pathway. Biochem. Biophys. Res. Commun. 338, 1299–1305.

72. Liu, N., Bezprozvannaya, S., Williams, A. H., Qi, X., Richardson, J. A., Basell-Duby, R., and Olson, E. N. (2008) microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. Genes Dev. 22, 3242–3254.

73. Hua, Y., Zhang, Y., and Ren, J. (2012) IGF-1 deficiency resists cardiac hypertrophy and myocardial contractile dysfunction. Role of microRNA-1 and microRNA-133a. J Cell Mol Med 16, 83–95.

74. Matsui, T., Li, L., Wu, J. C., Cook, S. A., Nagoshi, T., Picard, M. H., Liao, R., and Rosenzweig, A. (2002) Phenotypic spectrum caused by transgenic overexpression of activated Akt in the heart. J. Biol. Chem. 277, 22896–22901.

75. Matsui, T., Nagoshi, T., and Rosenzweig, A. (2003) Akt and PI 3-kinase signaling in cardiomyocyte hypertrophy and survival. Cell Cycle 2, 220–223.

76. Sanna, B., Bueno, O. F., Dai, Y. S., Wilkins, B. J., and Molkentin, J. D. (2005) Direct and indirect interactions between calcineurin-NFAT and MEK1-extracellular signal-regulated kinase 1/2 signaling pathways regulate cardiomyocyte gene expression and cellular growth. Mol. Cell. Biol. 25, 865–878.

77. Denayer, E., and Legius, E. (2007) What’s new in the neurocardiofacial cutaneous syndromes? Eur. J. Pediatr. 166, 1091–1098.

78. Tidyman, W. E., and Rauen, K. A. (2009) The RASopathies. Developmental syndromes of Ras/MAPK pathway dysregulation. Curr. Opin. Genet. Dev. 19, 230–236.

79. Tartaglia, M., Martinelli, S., Stella, L., Bocchinfuso, G., Flex, E., Cordeddu, V., Zampino, G., Burgt, I. V., Palleschi, A., Petrucci, T. C., Sorcini, M., Schoch, C., Foa, R., Emanuel, P. D., and Gelb, B. D. (2006) Diversity and functional consequences of germline and somatic PTPN11 mutations in human disease. Ann. J. Hum. Genet. 78, 279–290.

80. Roberts, A. E., Araki, T., Swanson, K. D., Montgomery, K. T., T. A., Joshi, V. A., Li, L., Yassin, Y., Tamburino, A. M., Molkentin, J. D., Kucherlapati, R. S. (2007) Germline gain-of-function mutations cause Noonan syndrome. Nat. Genet. 39, 70–75.

81. Schubbert, S., Zenker, M., Rowe, S. L., Böll, S., Klein, C., Bollag, G., van der Burgt, I., Musante, L., Kalscheuer, V., Webner, L. E., Nguyen, H., West, B., Zhang, K. Y., Sisternas, E., Rauch, A., Niemeyer, C. M., Shannon, K., and Kratz, C. P. (2006) Germline KRAS mutations cause Noonan syndrome. Nat. Genet. 38, 331–336.

82. Pandit, B., Sarkozy, A., Pennacchio, L. A., Carta, C., Oishi, K., Martinelli, S., Pogna, E. A., Schackwitz, W., Ustaszewska, A., Landstrom, A., Bos, J. M., Ommen, S. R., Esposito, G., Lepri, F., Faul, C., Munder, P., López Siguero, J. P., Tenconi, R., Selicorni, A., Rossi, C., Mazzanti, L., Torrente, I., Marino, B., Digilio, M. C., Zampino, G., Ackerman, M. J., Dallapiccola, B., Tartaglia, M., and Gelb, B. D. (2007) Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. Nat. Genet. 39, 1007–1012.

83. Wu, X., Simpson, J., Hong, J. H., Kim, K. H., Thavarajah, N. K., Backx, P. H., Neel, B. G., and Araki, T. (2011) MEK-ERK pathway modulation ameliorates disease phenotypes in a mouse model of Noonan syndrome associated with the Raf1(L613V) mutation. J. Clin. Invest. 121, 1009–1025.

84. Marin, T. M., Keith, K., Davies, B., Conner, D. A., Guha, P., Kalaitzidis, D., Wu, X., Lauriol, J., Wang, B., Bauer, M., Bronson, R., Franchini, K. G., Neel, B. G., and Kontaridis, M. I. (2011) Rapamycin reverses hypertrophic cardiomyopathy in a mouse model of LEOPARD syndrome-associated PTPN11 mutation. J. Clin. Invest. 121, 1026–1043.

85. Krenz, M., Gulick, J., Qiu, J., H. E., Colbert, M. C., Molkentin, J. D., and Robbins, J. (2008) Mediating ERK 1/2 signaling in congenital valve malformations in Noonan syndrome. Proc. Natl. Acad. Sci. U.S.A. 105, 18930–18935.

86. Nakamura, T., Colbert, M., Krenz, M., Gulick, J., Pratte, R., and Robbins, J. (2009) Noonan syndrome is associated with enhanced pERK activity, the repression of which can prevent craniofacial malformations. J. Clin. Invest. 121, 2123–2132.

87. Nakamura, T., Colbert, M., Krenz, M., Molkentin, J. D., Hahn, H. S., Dorn, G. W., 2nd, and Robbins, J. (2007) Mediating ERK 1/2 signaling rescues congenital heart defects in a mouse model of Noonan syndrome. J. Clin. Invest. 117, 2123–2132.

88. Nakamura, T., Colbert, M., Krenz, M., Molkentin, J. D., Hahn, H. S., Dorn, G. W., 2nd, and Robbins, J. (2009) Noonan syndrome is associated with enhanced pERK activity, the repression of which can prevent craniofacial malformations. Proc. Natl. Acad. Sci. U.S.A. 106, 18930–18935.

MicroRNA-378 Targets Ras Signaling in Cardiac Myocytes

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 288 • NUMBER 16 • APRIL 19, 2013

WITHDRAWN

February 27, 2017
Supplemental Information

A cardiac enriched microRNA, miR-378 blocks cardiac hypertrophy by targeting Ras-signaling

Raghu S. Nagalingam¹, Nagalingam R. Sundaresan², Mahesh P. Gupta², Dave Geenen¹, R. John Solaro¹, and Madhu Gupta¹

¹From the Department of Physiology and Biophysics and Center for Cardiovascular Research, University of Illinois, Chicago IL 60612

²Department of Surgery, Committee on Cellular and Molecular Physiology, University of Chicago, Chicago IL 60637

*Running title: MicroRNA – 378 targets Ras-signaling in cardiac myocytes
MicroRNA-378 targets Ras-signaling in cardiac myocytes

Table 1: Genomic location, sequence information of miR-378 isoforms as described for human in microRNA database. The seed sequence and its conservation in various isoforms is depicted in red, sequence variation is highlighted in black. Chr = Chromosome location.

| miR-378 isoforms | Sequence of mature miR-378 | Genomic location |
|------------------|---------------------------|------------------|
| hsa-miR-378a     | ACUGGACUUGGAGUCAGAAGG     | Chr 5, Intron 1 of PGC1β (sense) |
| hsa-miR-378b     | ACUGGACUUGGAGGCAGAA       | Chr 3, intron 1 of ATP2B2 (antisense) |
| hsa-miR-378c     | ACUGGACUUGGAGUCAGAAGAGUGG | Chr 10, Intergenic region |
| hsa-miR-378d-1   | ACUGGACUUGGAGUCAGAAA      | Chr 4, Intergenic region |
| hsa-miR-378d-2   | ACUGGACUUGGAGUCAGAAA      | Chr 8, Intron 1 of Pyruvate dehydrogenase phosphatase (antisense) |
| hsa-miR-378e     | ACUGGACUUGGAGUCAGGA       | Chr 5, Intron 1 of DOCK2 (sense) |
| hsa-miR-378f     | ACUGGACUUGGAGCAGAAG       | Chr 5, Intron 1 of DOCK2 (antisense) |
| hsa-miR-378g     | ACUGGACUUGGAGUCAGAAA      | Chr 10, Intergenic region |
| hsa-miR-378h     | ACUGGACUUGGAGUCAGGAGAAG   | Chr 4, Intergenic region |
| hsa-miR-378i     | ACUGGACUUGGAGUCAGAGAAG    | Chr 10, Intergenic region |

*WITHDRAWN*
**Figure S1:** MiR-378 inhibits PE-stimulated early activation of ERK1/2 in cardiomyocytes. Cells were transfected with mimic-C or miR-378, after 48 h cell media was changed to serum free. After 18 h cells were stimulated with PE (20 uM) for 5 or 15 min (n=3). * significant as compared to PE treated mimic-C.
Figure S2: Inhibition of miR-378 results in ANF induction under basal conditions and enhanced expression with PE-stimulation. A 3-fold induction was noted with 378-antimiR under basal conditions. Almost 3-fold higher expression of ANF was also noted following PE-treatment for 48 h in 378-antimiR transfected cells as compared to scramble-C (n=3). † significant as compared to vehicle treated scramble control, * significant when compared to PE treated control. For a representative radiogram please refer to Figure 7B.
Figure S3: Adenovirus, Ad-rasN17, expressing dominant negative mutant form of Ras, inhibits serum-stimulated AKT activation in cardiomyocytes. One of the known downstream effectors of Ras-signaling is phosphorylation of AKT. To test the effectiveness of Ad-rasN17 adenovirus (used in this study) in inhibiting Ras signaling in cardiomyocytes serum-stimulated AKT activation was measured in cardiomyocytes after infection with Ad-rasN17 or control adenovirus (Ad-blank). Ad-rasN17 but not Ad-blank significantly inhibited serum-stimulated activation of pAKT (n=3). † significant as compared to serum free (SF) Ad-blank control, * significant when compared to serum stimulated Ad-blank control.
Figure S4: Over expression of miR-378 reduces Grb2 protein expression and Grb2 mRNA levels in cardiomyocytes. A. Quantification of Grb2 protein levels with increasing doses of miR-378 (n=3). For a representative radiogram please refer to Figure 9B. B. Over-expression of miR-378 resulted in almost 50% reduction in the mRNA levels of Grb-2 as measured by real-time PCR (n=3). * Significant as compared to mimic-C control.
Figure S5: Grb2 in PE-stimulated hypertrophy of cardiomyocytes and in human heart failure: A. Quantification of Grb2 in PE treated cardiomyocytes. Expression of Grb2 is induced after PE treatment. Over-expression of miR-378 caused a significant reduction in Grb2 and it prevented PE-stimulated induction of Grb2. † significant in relation to non-treated cells, * significant in relation to corresponding controls transfected with mimic-C (n=2). For a representative radiogram refer to Figure 9C. B. Quantification of Grb2 expression in human samples of non-failing heart (NF1, NF2, NF3) and heart patients with non-ischemic cardiomyopathy (DCM1, DCM2, DCM3). Each bar is a mean ± SD of three replicate assays. * Significant in relation to non-failing hearts. Representative radiogram is shown in Figure 9D.
MicroRNA-378 targets Ras-signaling in cardiac myocytes

Figure S6: Knockdown of Grb2 by a duplex SiRNA antagonizes the effect of 378-antimiR on ANF expression under basal conditions and reduces PE-stimulated induction of fetal genes. A. Transfection of cardiomyocytes with 378-antimiR caused almost 12-fold induction in ANF protein levels when it was co-transfected with a negative control SiRNA whereas co-transfection with Grb2 SiRNA produced only 4.5-fold induction. There was no difference when Grb2 SiRNA or control SiRNA were co-transfected with scramble control. For a representative radiogram please refer to Figure 9F. † significant when compared to scramble control, * significant in relation to control siRNA. B. Real-time PCR analysis of hypertrophy markers in PE-stimulated cardiomyocytes following Grb2 inhibition. Cardiomyocytes were transfected with control SiRNA or Grb2 SiRNA and then treated with PE for 48 h. Cells transfected with control SiRNA treated with vehicle served as control. Data presented as mean ± SD. Each assay performed in triplicates (n=2). * significant in relation to vehicle treated control SiRNA and also with PE-stimulated control SiRNA.

WITHDRAWN
A Cardiac-enriched MicroRNA, miR-378, Blocks Cardiac Hypertrophy by Targeting Ras Signaling
Raghu S. Nagalingam, Nagalingam R. Sundaresan, Mahesh P. Gupta, David L. Geenen, R. John Solaro and Madhu Gupta

J. Biol. Chem. 2013, 288:11216-11232.
doi: 10.1074/jbc.M112.442384 originally published online February 27, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.442384

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2013/02/27/M112.442384.DC1

This article cites 87 references, 45 of which can be accessed free at
http://www.jbc.org/content/288/16/11216.full.html#ref-list-1

WITHDRAWN
February 27, 2017