Deficiency of Cardiomyocyte-specific MicroRNA-378 Contributes to the Development of Cardiac Fibrosis Involving a Transforming Growth Factor β (TGFβ1)-dependent Paracrine Mechanism*

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Background: MicroRNA-378, a cardiomyocyte-specific miRNA, is down-regulated during heart failure. Results: miR-378 inhibition induced TGFβ1 expression, which correlated with the activity of c-Fos, c-Jun, and Ras. Conditioned media of miR-378-depleted myocytes induced fibroblast activation by utilizing TGFβ1-dependent paracrine mechanisms.

Conclusion: miR-378 is a negative regulator of TGFβ1 and cardiac fibrosis.

Significance: miR-378 offers therapeutic potential for the management of heart failure.

This article has been withdrawn by the authors due to reuse of the U6 loading controls in the right and left panels of Fig. 4A and the GAPDH loading controls in Figs. 7D and 9F. Additionally, the journal investigated the IGF1R immunoblot in Fig. 5C and the collagen immunoblot in Fig. 6C. The authors were not able to provide the original data for the collagen immunoblot in Fig. 6C.

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‡1 The abbreviations used are: miRNA, microRNA; LNA, locked nucleic acid; SCR, scrambled; Ang II, angiotensin II; FN, fibronectin; CTGF, connective tissue growth factor; HW, heart weight; IGF1R, IGF1 receptor; TAC, transverse aortic constriction; CM, conditioned media; Dn, dominant negative; GSIII, glycogen synthase kinase 3; LV, left ventricle; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; MHC, myosin heavy chain.
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hypothesis. Functional analysis using both gain and loss of function approaches have demonstrated that by targeting distinct miRNAs in myocytes and in fibroblasts, miRNAs either promote (pro-hypertrophic) or prevent (anti-hypertrophic) development of hypertrophy and fibrosis (for review see Ref. 4).

It is now apparent that within the heart some miRNAs specifically function to regulate fibroblast proliferation, differentiation, and induction of fibrosis. For example, miR-29, miR-133, and miR-30 are known to suppress expression of collagen and extracellular matrix proteins. Although miR-29 is expressed in fibroblasts, miR-133 is in myocytes, and miR-30 is present both in myocytes and fibroblasts (5–8). Interestingly, there is evidence that miR-29 and miR-30 are secreted from cardiac fibroblasts and influence cardiomyocyte growth. Conditioned medium of miR-29-overexpressing cardiac fibroblasts attenuated cardiomyocyte growth, whereas media of miR-30-expressing fibroblasts resulted in the induction of cardiomyocyte growth (9).

We and others have recently demonstrated that miR-378 (also known as miR-378–3p) is primarily expressed in cardiac myocytes, and its expression level is reduced in human failing hearts as well as in various experimental models of cardiac hypertrophy (10–13). Overexpression of miR-378 protected cardiomyocytes from undergoing hypertrophy (12) and prevented cardiac dysfunction induced by pressure overload in mice (13). Based on these results, miR-378 is considered an anti-hypertrophic miRNA. In contrast to the anti-hypertrophic effects of miR-378 overexpression, whole body overexpression of miR-378 also showed beneficial results on induced changes in systemic energy metabolism (14). The cardiac function and phenotype ratios of these animals were, however, not described.

In the current study we propose a protective role of miR-378 in both cardiac myocytes and fibroblasts. We demonstrate that inhibition of Ras signaling by locked nucleic acid (LNA)-modified oligonucleotides promotes cardiomyocyte hypertrophy and exaggerates angiotensin II–induced adverse cardiac remodeling and dysfunction in mouse hearts. Depletion of miR-378 alone led to increased expression and secretion of proinflammatory cytokine, TGFβ1, by involving Ras-signaling-dependent mechanisms and AP-1 transcription factor activities. Our study thus demonstrates a new protective role of miR-378 in the process of cardiac remodeling during stress and that the presence of miR-378 is critical in maintaining cardiac cellular homeostasis.

**EXPERIMENTAL PROCEDURES**

All animal protocols were reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee.

**Delivery of LNA AntimiR**—The 378-antimiR oligonucleotides were synthesized at Exiqon Inc as fully phosphorothioated oligonucleotides with LNA modifications as outlined in Fig. 1A. The LNA control (SCR) was a Caenorhabditis elegans-specific miRNA (15). Adult C57BL/6 mice were injected with LNA-antimiR or LNA-SCR (70 mg/kg, intraperitoneally) in a similar volume of saline on three consecutive days.

**Cardiac Hypertrophy**—Cardiac hypertrophy was induced in adult C57BL/6 mice by transverse aortic constriction for 4 weeks as described before (16) and by angiotensin II (Ang II, 1 mg/kg/day) for 1 week. Subcutaneously implanted osmotic mini pumps (ALZET model 1007D) were used to deliver Ang II at a flow rate 0.5 μl/h/day.

**Echocardiography**—Transthoracic echocardiography in mice was performed at the University of Illinois Center for Cardiovascular research core facility in a blinded fashion under isoflurane (~1%) anesthesia with a VisualSonics Vevo 770 instrument using a 30-MHz high frequency transducer as described (17, 18).

**Cell Culture, Transfection, and Treatments**—Primary cultures of cardiomyocytes and fibroblasts were prepared from neonatal Sprague-Dawley rats as described before (10). Fibroblasts obtained during the pre-plating step of cardiomyocyte culture preparation were grown in Dulbecco’s modified Eagle's medium supplemented with penicillin/streptomycin and 10% fetal bovine serum. Fibroblasts of 2nd or 3rd passage were used all throughout the experiment. Cells were treated with phenylephrine (20 μM) or Ang II (100 nM) for the indicated time periods in serum-free DMEM medium.

For overexpression studies cells were transfected with 25 nM synthetic miR-378–3p (Ambion Inc.). A sequence-specific scramble control (each at 10 nM). After 24 h of transfection, cells were infected at a multiplicity of infection of 10 plaques/cm². For knocking down Ras signaling cardiomyocytes were infected with lentivirus containing the Dn allele of c-Ras (N17; 1031; Vector Biolabs) and Ad-CMV-c-Jun (Dn) (1046; Vector Biolabs) were used. For control, adenovirus Ad-CMV-GFP was used.

**Preparation of Conditioned Medium**—Cardiomyocytes were transfected with either LNA-modified miR-378-antimiR or scramble control (each at 10 nM). After 24 h of transfection, medium was changed to complete growth medium, and cells were incubated for an additional 48–72 h. During this period, 25% of the medium was collected every 24 h and replaced with the fresh medium. At the end of the incubation period medium was collected, and all collected media were pooled and labeled as conditioned medium. This was centrifuged at low speed to remove cell debris, frozen in aliquots, and used as needed.

**Real-time PCR and Northern Blot Analysis**—Total RNA was extracted and resolved on a urea gel for Northern analysis using standard techniques. The RNA was transferred to nitrocellulose membranes and hybridized with radiolabeled microRNA-specific probes; U6 was used as a normalization control. For real-time PCR total RNA was reverse-transcribed using standard protocols. Expression of miR-378, U6, ANF, and βMHC were analyzed by using Taqman assays, and the remaining miRNAs were analyzed by SYBR Green-based assays. Primer sequences are available upon request.

**Tissue Histology and Immunostaining**—Hearts were isolated and perfusion-fixed and processed for light microscopy. Tissue embedding and staining of heart sections were performed by the histology core facility at University of Chicago. Cell imaging...
was performed on a Bio–Rad Laser Sharp 2000 system using a 40× objective (Zeiss).

Hearts were excised, perfused with saline, and fixed in formalin. After dehydration in graded ethanol solutions, tissues were cleared with xylene and processed for embedding. The paraffin-embedded hearts were sectioned at 4 μm and subsequently stained with wheat germ agglutinin coupled to tetramethylrhodamine isothiocyanate (Sigma) for detection of cell size as described earlier (19). Left ventricular myocyte cross-sectional area was measured on sections of mid-free wall of the left ventricle. Suitable cross-sections were defined as having nearly circular capillary profiles and circular to oval myocyte cross-sections. The outer borders of the myocytes were traced, and myocyte areas were calculated with NIH ImageJ software (rsbweb.nih.gov). Approximately 200 cells were counted per sample, and the average was used for analysis.

Masson’s trichrome staining was performed to detect collagen fiber density using standard protocols (19). The collagen fraction (stained with aniline blue in Masson’s trichrome-stained sections) was calculated as blue-stained collagen fiber area divided by total area of the visual field. Analysis was performed in a minimum of 5 hearts for each experimental group with at least 5 replicates of each sample, and 10 visual fields were measured in each replicate. Fibroblasts (10,000–20,000)

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**FIGURE 1. Inhibition of miR-378 by LNA-modified anti.miR.** A, design of LNA-modified anti.miR and sequence alignment of human miR-378 showing high sequence homology among various isoforms of miR-378. Highlighted in gray is seed sequence, underlined nucleotides denote divergence from miR-378a, and the dotted line marks the complimentary sequences in anti.miR. Asterisks identify LNA modified nucleotides in 378-antimiR (antimiR) and in the scramble control (SCR) oligonucleotides. B, dose-dependent inhibition of miR-378 by anti.miR by Northern analysis using 5 μg of total RNA. Mice were injected on three consecutive days, and analysis was performed after 7 days. The membrane was sequentially hybridized with miR-1 for testing specificity of inhibition and with U6 for loading control. C, Northern analysis of miR-378 expression using 5 μg of total RNA control (SCR) and anti.miR (70 mg/kg)-treated mouse hearts. Each lane represents a biological replicate. D, measurement of miR-378 expression by real-time PCR. Data are presented as the mean ± S.D. of 8–10 hearts. *, significant compared with SCR control. E and F, Northern analysis using 20 μg total RNA (skeletal muscle) and 50 μg of RNA (for kidney and liver).
were plated on cover slips and processed for immuno-staining for collagen and vimentin as per the protocols described previously (10, 12).

**Western Blot Analysis**—Western blotting was performed using standard protocols.

**ELISA Assay**—A multiplex ELISA sandwich assay was performed for measuring the levels of cytokines in the conditioned media by using Rat Oxidative ELISA Strip (Signosis Inc.) as per the manufacturer’s protocol.

**Antibodies**—The following antibodies were used in this study: Akt1 (C73H10; Cell Signaling), P-Akt (Ser-473; Cell Signaling), P-p44/42MAPK (Thr-202/Tyr-204; Cell Signaling), P-GSK3β (S9; Cell Signaling), P-p70-S6 kinase (Thr-389; Cell Signaling), IGFR1 (3027; Cell Signaling), P-glycogen synthase (Ser-641; Cell Signaling), glycogen synthase (15B1; Cell Signaling), β-actin (SC-1616; Santa Cruz), P-c-Jun (sc-641; Cell Signaling), c-Jun (sc-1694; Santa Cruz), fibronectin (sc-9068; Santa Cruz), c-Fos (sc-253; Santa Cruz), P-STAT3 (Ser-235/Ser-255; Cell Signaling), HRP-conjugated anti-mouse (A2304; Sigma), HRP-conjugated anti-goat (sc-2020; Santa Cruz), HRP-conjugated anti-rabbit (7074; Cell Signaling), HRP-conjugated anti-rabbit (T-4014; Peninsula Laboratories), anti-collagen type I rabbit (234167; Calbiochem), anti-hTGFβ1 mouse (MAB240; R&D Systems), anti-vimentin mouse (V5255; Sigma), donkey anti-goat IgG-Alexa Fluor 594 (A11058; Invitrogen), and donkey anti-rabbit IgG-Alexa Fluor 594 (A21207; Invitrogen).

**Statistical Analysis**—Data are expressed as the mean ± S.D. Student’s t test and one-way analysis of variance (ANOVA) were used for statistical analysis. Echocardiography data were analyzed with ANOVA followed by the Bonferroni post hoc test in Graphpad Prism. Differences with a p value of <0.05 were considered statistically significant.

**RESULTS**

**Inhibition of Cardiac miR-378 by AntimiR**—To understand the role of miR-378 in cardiac pathophysiology, we used a LNA-modified oligonucleotide to knockdown endogenous miR-378 in mice. LNA-based oligonucleotide modification has been shown to impart resistance to nuclease degradation *in vivo* with minimal biological toxicity (20). This approach has been used by several investigators for the therapeutic inhibition of microRNAs *in vivo*. A 25-bp phosphorothioate oligonucleotide containing 46% LNA nucleotides was used to target miR-378. AntimiR was designed to target the seed sequence regions (Fig. 1A).

We found that antimiR caused a dose-dependent inhibition of miR-378 expression in the heart, whereas SCR control had no effect (Fig. 1B). The specificity of antimiR-mediated targeting

**TABLE 1**

**Animal characteristics after antimiR injections**

| Parameters | Scramble group (n = 8) | AntimiR group (n = 8) |
|------------|------------------------|-----------------------|
| Body weight (g) | 22.5 ± 0.9 | 23.0 ± 0.5 |
| HW weight (mg) | 110 ± 5 | 115 ± 3 |
| Tibia length (mm) | 16.9 ± 0.2 | 17.1 ± 0.2 |
| HW/BW (mg/g) | 4.89 ± 0.1 | 4.92 ± 0.1 |
| HW/TL (mm/mm) | 6.37 ± 0.2 | 6.42 ± 0.2 |

*p < 0.05 when compared to scramble control.

**TABLE 2**

**Effect of miR-378 inhibition on cardiac function by echocardiography in mice before (basal) and 7 days after scramble or antimiR injection**

| Parameters | Basal (n = 8) | AntimiR (n = 8) |
|------------|--------------|----------------|
| Age | 182 ± 3 | 186 ± 3 |
| Body weight (g) | 21.98 | 22.38 |
| Heart rate (beats/min) | 485 ± 25 | 458 ± 38 |
| LV dimensions | | |
| LVAWd (mm) | 0.68 ± 0.04 | 0.65 ± 0.02 |
| LVAWd (mm) | 0.97 ± 0.04 | 0.90 ± 0.01 |
| LVPWd (mm) | 0.65 ± 0.03 | 0.69 ± 0.04 |
| LVPWd (mm) | 0.96 ± 0.05 | 0.95 ± 0.06 |
| LVdDd (mm) | 3.73 ± 0.07 | 3.91 ± 0.08 |
| LVdDd (mm) | 2.78 ± 0.06 | 2.97 ± 0.05 |
| RWT | 0.359 ± 0.02 | 0.347 ± 0.008 |
| LV mass (mg) | 65.95 ± 7.5 | 64.8 ± 5.2 |
| Corrected LV mass (mg) | 52.76 ± 6 | 51.84 ± 4.4 |

* *p < 0.05 when compared to basal or SCR control.*
of miR-378 was validated by probing the same membrane for expression of miR-1 and miR-208a, which remained unchanged (Fig. 1, B and C; data are not shown for miR-208a). For subsequent experiments, we used a 70 mg/kg dose of antimiR that consistently decreased miR-378 expression by ~70% (Fig. 1, C and D). We also analyzed tissue samples from the skeletal muscle, liver, and kidney of antimiR-injected animals and found a significant inhibition of miR-378 expression in these tissues (Fig. 1, E and F). As compared with the heart, the kidney, and liver had much lower basal expression of miR-378, consistent with previous findings (10, 14); however, in our study the higher molecular weight antimiR-miR-378 duplex was observed only in the kidney, the tissue that exhibited least expression levels of miR-378.

**MiR-378 Depletion Induces Cardiac Hypertrophy in Mouse Hearts**—We have previously shown that miR-378 is down-regulated in experimental models of cardiac hypertrophy as well as in human failing hearts (11, 12), a finding also confirmed by others (13). To investigate whether miR-378 knockdown alone is sufficient to modulate cardiac gene expression in vivo, mice were injected with antimiR as described above. After 7 days of injection, the animals were sacrificed, and the hypertrophic response was determined by measuring the heart-to-body weight ratio, cardiac chamber dimensions, cardiomyocyte size, and expression of hypertrophy markers (ANF, BNP, α-skeletal actin, β-MHC). Fibrotic response of the heart was evaluated by measuring expression levels of collagen isoforms (Col1α1 and Col3α1), fibronectin (FN), connective tissue growth factor (CTGF), and by Masson’s trichrome staining for collagen fibers. The cardiac function was assessed by M-mode echocardiography and by Doppler flow imaging. We found that inhibition of miR-378 had no significant effect on the mouse body weight or on the gross morphology of the heart. Measurements of HW/body weight, and HW/tibia length showed significantly less...
higher ratios in antimiR than in SCR controls (Table 1). M-mode echocardiography revealed increased LV mass, anterior wall thickness, and relative wall thickness in antimiR-injected animals compared with pre-injection measurements. These parameters did not change with SCR (Table 2). AntimiR administration also resulted in increased cardiomyocyte cross-sectional area (Fig. 2, A and B) and increased expression of hypertrophy markers, ANF, BNP, and α-skeletal actin. However, there was no change in β-MHC mRNA levels (Fig. 2C). These effects of AntimiR were associated with increased phosphorylation of AKT and GSK3β, which correlated with reduced phosphorylation of GSK3β substrate, glycogen synthase, thus suggesting increased activity of pro-hypertrophic AKT and reduced anti-hypertrophic GSK3β-signaling (Fig. 2, D and E). We also observed de-repression of previously identified (10–13) direct targets of miR-378, IGF1R, and GRB-2 in antimiR-treated hearts (Fig. 3, A and B). These results thus indicated that miR-378 depletion induces cardiac hypertrophy and that miR-378 is critically involved in maintaining lower expression levels of IGF1R, GRB-2, and in the regulation of AKT-GSK3β signaling under basal conditions.

We next examined the effects of miR-378 depletion on systolic and diastolic function of the mouse heart. The results showed that systolic function is preserved in antimiR-injected animals as there was no difference in fractional shortening or ejection fraction before and after antimiR treatment and between antimiR-injected and SCR control animals. Measurements of diastolic function by flow Doppler of trans-mitral flow showed that antimiR treatment produced relatively lower early ventricular filling with higher E-wave amplitude and reduced deceleration time (E DT). The late ventricular filling A-wave amplitude or E/A ratios were, however, not significantly altered by antimiR (Table 2). Thus, miR-378 depletion over a 7-day treatment period caused only mild diastolic abnormalities.

Another consequence of induction of pathologic hypertrophy is the development of fibrosis. We, therefore, performed Mason’s trichrome staining of serial heart sections, which showed increased formation of collagen fibers in the antimiR treated hearts compared with SCR controls (Fig. 3, A and B). Because we and others have previously shown that miR-378 is primarily expressed in cardiomyocytes (10, 13), observing changes in the expression of fibrotic markers in antimiR-treated hearts with mostly preserved cardiac function was an unexpected finding. For further confirmation we compared mRNA levels of fibrotic markers Col1α1, Col3α1, FN, and CTGF by real-time PCR and found significant induction in all four gene transcripts in antimiR-treated than SCR control hearts. Similarly, there was increased protein expression of FN and Collagen1 in antimiR-treated hearts (Fig. 3, C–E). These data thus demonstrated that miR-378 depletion induces cardiac fibrosis in mouse hearts.

**MiR-378 Depletion Exaggerates Angiotensin II-induced Cardiac Hypertrophy, Fibrosis, and Cardiac Dysfunction in Mice—** To further evaluate the role of miR-378 in cardiac remodeling, we studied the effect of miR-378 depletion in combination with
the hypertrophy agonist, Ang II. In our previous studies, 3 mg/kg/day infusion of Ang II for 2 weeks produced extensive fibrosis and severe cardiac dysfunction with reduced fractional shortening (22). In this study we used a low dose of Ang II (1 mg/kg/day) for 1 week to avoid severe adverse remodeling and to study the possible additive effects of miR-378 depletion. We found that at this dose, Ang II produced almost 15–20% increase in HW/tibia length ratio accompanied with significant induction of hypertrophy and fibrotic markers but without altering ejection fraction and fractional shortening, consistent with other reports using a similar dose (23, 24).

We previously reported down-regulation of miR-378 during isoproterenol infusion and pressure-overload-induced hypertrophy of the heart (12). In the current study we found that Ang II infusion also reduced miR-378 levels by ~40% when administered alone and ~85% when combined with antimiR (Fig. 4, A and B). In antimiR + Ang II animals there was a larger increase in HW/tibia length ratio, cross-sectional area of cardiomyocytes, and mRNA levels of hypertrophy markers, ANF, BNP, α-skeletal actin, and β-MHC, than in SCR + Ang II-treated controls (Fig. 4, C–E). We also measured elements in the Ang II-induced signaling cascade and found higher activation of prohypertrophic molecules such as pS6K, pERK1/pERK2, and pAKT in antimiR-treated hearts. The kinase activity of the antihypertrophic signaling molecule, GSK3β, was reduced in antimiR + Ang hearts, as measured by the increased levels of

### FIGURE 4. Inhibition of miR-378 enhances angiotensin II-induced cardiac hypertrophy.

A. Northern analysis of miR-378 expression in individual hearts using 10 μg of total RNA. The left panel shows inhibition of miR-378 by angiotensin infusion (SCR + Ang) in relation to saline pumps (SCR + Sal) in scramble (SCR) control animals, and the right panel shows miR-378 inhibition by antimiR (AntimiR + saline (Sal)) and additive inhibition of miR-378 with combined antimiR and angiotensin II treatment (20 μg of total RNA was used). U6 served as a loading control.

B. Quantification of miR-378 expression by real-time PCR.

C. Heart weight/tibia length ratios in various groups of animals as indicated. D. Real-time PCR analysis for hypertrophy marker mRNA levels in various groups of animals. E. Wheat germ agglutinin-stained images of cross-section of cardiac tissues and cell size measurements in indicated groups of animals. Measurements in all figures are presented as the mean ± S.D. of 6–8 animals.

* indicates significant difference when compared with SCR controls; # indicates significant from angiotensin-treated SCR control.
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pGSKβ and reduced phosphorylation of the substrate, glycogen synthase (Fig. 5, A and B). The de-repression of miR-378 targets, IGF1R and GRB-2, was also exacerbated by combined treatment of Ang II with anti-miR than Ang II with SCR (Fig. 5C). Overall our findings indicated that miR-378 inhibition augments adverse cardiac remodeling induced by Ang II in mouse heart.

By M-mode echocardiography, anti-miR + Ang II hearts showed a significantly larger increase in LV wall thickness particularly of the anterior wall, reduced LV cavity dimensions, and a larger increase in the calculated LV mass than SCR + Ang II hearts (Table 3). By trans-mitral flow Doppler measurements, as compared with their base-line values, SCR + Ang II hearts showed reduced E/A ratio and prolonged E-wave deceleration time, whereas anti-miR + Ang II hearts showed increased E-wave amplitude with a shortened E wave deceleration time and significantly higher E/A ratio. Collectively echocardiography data suggested impaired myocardial relaxation in SCR + Ang II, which deteriorated further to a restrictive diastolic dysfunction in anti-miR + Ang II group (Table 3).

The pro-fibrotic activity of Ang II was significantly exaggerated when combined with anti-miR than with SCR. This was reflected in a 1) significantly larger collagen fiber staining area, 2) higher induction of Col1α1, Col3α1, FN, and CTGF mRNAs, and 3) higher protein levels of fibronectin and collagen1 in anti-miR + Ang II-treated hearts than the corresponding control hearts (Fig. 6, A–E). For further confirmation of pro-fibrotic effects of miR-378 inhibition, we tested fibrotic marker expression in another model of cardiac hypertrophy induced by transverse aortic constriction (TAC) for 4 weeks. Again we found greater induction of fibrotic markers in anti-miR + TAC hearts than SCR + TAC hearts (Fig. 6F). From these data it became

FIGURE 5. Hypertrophy signaling cascade and expression of direct targets of miR-378 in Ang II-stimulated and anti-miR treated mouse hearts. A, Western analysis and quantification of phosphorylated 56 kinase (normalized to GAPDH) and pERK1/2 (normalized to ERK2) in mouse hearts of the indicated experimental groups. B, Western analysis and quantification of pAKT (normalized to total AKT) and pGSK3β and p-glycogen synthase (normalized to GAPDH) in heart samples of various experimental groups. C, Western analysis of miR-378 targets GRB-2 and IGF1R after angiotensin II infusion in control and anti-miR-treated mouse hearts. GAPDH was used as a loading control. In A–C, the same membrane was sequentially used to probe with indicated antibodies after stripping the membrane. Each bar represents the mean ± S.D. of n = 5–7 animals. The p prefix denotes the phosphorylated form. *, significant (p < 0.05) when compared SCR + saline control; #, significant compared with SCR + angiotensin group.
TABLE 3

Effect of miR-378 inhibition on angiotensin-induced changes in echocardiographic measurements of left ventricular wall thickness, chamber dimensions, and systolic and diastolic function by two-dimensional, M-mode, conventional Doppler, and tissue Doppler imaging.

| Parameters                        | Basal (n = 6) | SCR + Ang (n = 6) | Basal (n = 8) | AntimiR + Ang (n = 8) |
|-----------------------------------|---------------|------------------|--------------|----------------------|
| **Animal characteristics**        |               |                  |              |                      |
| Age (days)                        | 185 ± 3       | 192 ± 2          | 182 ± 5      | 189 ± 8              |
| Body weight (g)                   | 21 ± 0.17     | 24.225           | 22 ± 0.16    | 24.30 ± 0.79         |
| Heart rate (beats/min)            | 439 ± 20      | 465 ± 30         | 448 ± 28     | 453 ± 32             |
| **LV dimensions**                 |               |                  |              |                      |
| LVWAd (mm)                        | 0.69 ± 0.042  | 0.92 ± 0.028a    | 0.66 ± 0.032 | 1.26 ± 0.02ab        |
| LVWAs (mm)                        | 0.90 ± 0.022  | 1.2 ± 0.010a     | 0.88 ± 0.025 | 1.39 ± 0.015a        |
| LVVPd (mm)                        | 0.60 ± 0.010  | 0.82 ± 0.032a    | 0.64 ± 0.010 | 0.876 ± 0.012a       |
| LVVPs (mm)                        | 0.84 ± 0.028  | 1.09 ± 0.034a    | 0.90 ± 0.006 | 1.16 ± 0.013a        |
| LVDD (mm)                         | 3.91 ± 0.07   | 3.78 ± 0.07      | 4.02 ± 0.03  | 3.74 ± 0.059a        |
| LVDDs (mm)                        | 3.01 ± 0.042  | 2.96 ± 0.095     | 3.13 ± 0.063 | 2.73 ± 0.054a        |
| LV mass (mg)                      | 69.25 ± 8.8   | 97.8 ± 6.5a      | 72.77 ± 9.33 | 109.5 ± 5.78a        |
| Corrected LV mass (mg)            | 55.4 ± 7.04   | 78.24 ± 5.2a     | 58.21 ± 7.46 | 87.6 ± 4.62ab        |
| **Diastolic function**            |               |                  |              |                      |
| E (mm/s)                          | 700 ± 15      | 590 ± 28.5a      | 680 ± 27     | 898 ± 19ab           |
| A (mm/s)                          | 390 ± 16      | 424 ± 17.8       | 374 ± 13.8   | 310 ± 17.9ab         |
| E DT (ms)                         | 27.33 ± 0.54  | 31.0 ± 1.2a      | 26.2 ± 0.89  | 18.1 ± 0.77ab        |
| E/A (ratio)                       | 1.82 ± 0.16   | 1.39 ± 0.07a     | 1.81 ± 0.14  | 2.90 ± 0.19ab        |
| **Systolic function**             |               |                  |              |                      |
| EF (%)                            | 47.3 ± 2.8    | 48.3 ± 6.0       | 54.23 ± 5.1  |                      |
| FS (%)                            | 23.2 ± 2.0    | 21.6 ± 2.4       | 27.0 ± 2.9   |                      |

*a p < 0.05 when compared to the basal level.

*b p < 0.05 compared to angiotensin treated scramble (SCR) group.

Effect of miR-378 inhibition on angiotensin-induced changes in echocardiographic measurements of left ventricular wall thickness, chamber dimensions, and systolic and diastolic function by two-dimensional, M-mode, conventional Doppler, and tissue Doppler imaging.

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apparent that miR-378 inhibition enhances cardiac fibrosis induced by Ang II as well as by pressure overload.

MiR-378 Depletion Induces TGFβ Expression and Fibroblast Differentiation in Mouse Hearts—To probe into the mechanistic basis of the enhanced TGFβ expression and fibroblast differentiation, we performed multiplex ELISA assay. Results showed significantly higher levels of TGFβ1, TGFβ2, and TGFβ3 in antimiR-injected mouse hearts compared to the scramble control group (Fig. 7, data are not shown for other TGFβ-signaling components). Additionally, we examined mRNAs of TGFβ1, TGFβ2, CTGF, and FN in other tissues such as liver, kidney, and skeletal muscle tissues of antimiR-injected animals. We found higher levels of these mRNAs in kidneys of antimiR-injected animals; the induction of these transcripts in the liver and skeletal muscle tissues was not as robust and consistent as observed in kidneys (Fig. 8, A–D). These observations collectively indicated that miR-378 is a negative regulator of TGFβ1 in cardiomyocytes and its depletion results in the increased synthesis and release of TGFβ1 from cardiomyocytes and that the impact of miR-378 inhibition could be observed in remote tissues such as kidneys.

In the next series of experiments we examined whether conditioned media (CM) from miR-378-depleted cardiomyocytes is capable of modulating cardiac fibroblast activation. We prepared CM from miR-378-depleted cardiomyocytes (antimiR-CM) after transfection with 378-antimiR. CM of SCR-transfected cardiomyocytes (SCR-CM) was used as a control. The effect of CMs was tested on the activation of TGFβ signaling in cardiac fibroblasts by measuring SMAD2/3 phosphorylation, a critical step for transcriptional activation of fibrotic gene program. We observed that treatment of fibroblasts with antimiR-CM, but not with SCR-CM, triggered phosphorylation of SMADs (Fig. 9A). We also found that treatment of fibroblasts with anti-miR-CM alone was sufficient to induce mRNAs of fibrotic markers (FN, ColIα1, Col3α1, and CTGF; Fig. 9B) as well as it...
promoted collagen and fibronectin protein expression induced by Ang II (Fig. 9, C–E).

To gain further support for these findings, we took advantage of the ability of adrenergic stimulation of cardiomyocytes to induce TGFβ1 expression (25). We tested the combined effect of miR-378 inhibition and adrenergic stimulation of cardiomyocytes with phenylephrine on cardiac fibroblast activation. The results showed that anti-miR-CM of phenylephrine-stimulated cardiomyocytes further enhanced fibronectin expression than the SCR-CM of similarly stimulated myocytes (Fig. 9, D and E), thus again supporting pro-fibrotic activity of anti-miR-CM involving TGFβ1. To further confirm involvement of TGFβ1 in anti-miR-mediated fibrotic response, we included a TGFβ1-neutralizing antibody during cardiac fibroblasts incubation with anti-miR-CM. We found that TGFβ1-neutralizing antibody, but not a control antibody, counteracted anti-miR-CM-stimulated induction of FN, collagens, and CTGF mRNAs as well as it reduced protein expression of FN (Fig. 9, F and G). These data collectively established that inhibition of miR-378 in cardiomyocytes caused increased synthesis and
release of TGFβ1, which consequently led to the activation of cardiac fibroblasts and induction of fibrotic gene expression.

TGFβ1 is known to stimulate its own gene promoter via enhancing transcriptional activity of AP-1 (c-Fos-c-Jun complex) and Ras-signaling in a positive feed-forward regulatory mechanism (26–28). In our published report we demonstrated that in cardiomyocytes inhibition of miR-378 led to induction of Ras activity (12). In this study we also observed increased Ras activity in antiimiR-treated mouse hearts (data not shown). We, therefore, asked whether 378-antimiR-stimulated TGFβ1 expression in cardiomyocytes could be mediated by activation of AP-1 and/or induced Ras-signaling. To this end we first investigated the effect of antiimiR on c-Fos and c-Jun expression. We found that in cells where miR-378 was depleted, there was increased expression of both of these factors. This was further confirmed when the same membrane was probed for c-Jun phosphorylation, an indicator of c-Jun activity (Fig. 10A). We next tested involvement of Ras or c-Jun in the antiimiR-stimulated induction of TGFβ1 by using adenovirus vectors expressing either dominant negative N17-Ras or dominant negative c-Jun and measuring pc-Jun. The results showed that antiimiR-mediated activation of c-Jun was abolished by expression of Dn-Ras as well as by Dn-c-Jun. AntimiR-mediated induction of TGFβ1 mRNA was also abrogated in cardiomyocytes expressing these Dn-adenoviruses but not in those infected with a control adenovirus. Furthermore, a chemical inhibitor of AP-1,
Tanshinone, which blocked antimiR-induced c-Jun activation, also inhibited antimiR-mediated TGF-β1 induction (Fig. 10, B and C). These data thus demonstrated that activation of Ras-signaling and c-Fos and c-Jun activities significantly contribute to the induction of TGF-β1 resulting from miR-378 depletion in cardiac myocytes.

DISCUSSION

Data presented in the current study define a novel regulatory role of miR-378 in the maintenance of cardiac cellular homeostasis and control of cardiac fibroblast activation. We believe that miR-378 is a cardio-protective miRNA. In pathological conditions, when miR-378 levels are depleted, cardiomyocytes synthesize and release TGF-β1 and sensitize cardiac fibroblasts to pathological stimuli. Based on our findings reported here, a working model illustrating miR-378-mediated paracrine regulation of cardiac fibrosis is outlined in Fig. 11.

Several lines of experimental evidences are presented here to support that miR-378 is a negative regulator of TGF-β1 and cardiac fibrosis involving a paracrine mechanism. 1) Systemic inhibition of miR-378 by a LNA-modified antimiR alone led to induction of TGF-β1 mRNA and protein levels and stimulated fibrotic gene expression in mouse hearts. 2) When miR-378 depletion was combined with pro-fibrotic stimuli such as angiotensin II or transverse aortic constriction, a synergistic induction of cardiac fibrosis was observed in mouse hearts. 3) Depletion of miR-378 in cardiomyocytes induced TGF-β1 expression, whereas replenishment of miR-378 by a miR-378-mimic counteracted this effect. 4) Conditioned media of miR-378-depleted cardiomyocytes exhibited higher levels of TGF-β1. 5) Cardiac fibroblasts when treated with conditioned media of miR-378-depleted cardiomyocytes showed activated phenotype, which was suppressed by a TGF-β-neutralizing antibody. 6) We show that induction of TGF-β1 expression in miR-378-depleted cardiomyocytes largely depended on the activation of Ras signaling and on the expression of AP-1 transcription factors. To the best of our knowledge, our study is the first report demonstrating the ability of a cardiomyocyte-specific microRNA to regulate activation of cardiac fibroblasts by indirect modulation of TGF-β1 synthesis and release from cardiomyocytes.

Because induction of fibrosis is a common characteristic of many diseases, understanding the molecular regulation of fibroblast activation is of intense research interest. In the heart, development of fibrosis is a pathological feature associated with a variety of cardiomyopathies including myocardial infarction, hypertension, cardiac hypertrophy, and aging. Many studies suggest that cardiac muscle and non-muscle cells communicate through exchange of a variety of secreted proteins, growth factors, and hormones, which together form a complex regulatory network. Perturbations
in this regulatory network by pathological factors lead to activation and differentiation of fibroblasts into myofibroblasts, which synthesize excessive extracellular matrix proteins leading to the development of tissue fibrosis.

In experiments with primary cultures of cardiomyocytes as well as in in vivo mouse hearts, we found that inhibition of miR-378 led to increased expression of TGFβ. TGFβ1 is a prominent example of a secreted cytokine that is produced both by cardiomyocytes and cardiac fibroblasts and has been considered a central mediator of tissue fibrosis in the heart (29, 30). Its expression and signaling activity are significantly increased in experimental models of cardiac hypertrophy and human heart disease (30, 31). In our study we found that the conditioned medium of miR-378-depleted cardiomyocytes had higher TGFβ levels and induced fibroblast gene expression that was blocked by a TGFβ1 neutralizing antibody. Another cardiomyocyte-specific miRNA, miR-133 has also shown anti-fibrotic properties (32). Genetic deletion of miR-133 resulted in the induction of cardiac fibrosis. These effects involved direct targeting of TGFβ1 and also of collagen mRNA (33–35), although mechanistic details on targeting of fibroblast gene by a cardiomyocyte-specific miRNA were not addressed in these studies. In our study we observed no binding site of miR-378 in the 3′-UTR of TGFβ mRNA. We found that miR-378-antimiR
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Of note, activation of the Ras signaling pathway is also known to induce c-Jun phosphorylation and its transcriptional activity (36). A role of Ras GTPases in inducing cardiac hypertrophy is fairly established (37). It has been shown that besides controlling cell growth, Ras GTPases also regulate TGFβ/SMAD signaling but in a cell-context dependent manner. In epithelial cells, overexpression of constitutively active Ras blocked nuclear accumulation of SMADs, whereas in kidney mesangial cells, it promoted Smad3-dependent processes (28, 38, 39). In our study, besides heart, we also found higher up-regulation of Ras in kidneys than the liver or skeletal muscles of antimiR-treated mice. In this regard it should be noted that liver and liver exhibit considerably lower expression levels of miR-378 than skeletal muscle and heart tissues. AntimiR treatment induced fibrotic gene expression only in kidneys and in cardiac tissues, suggesting that it is not the absolute expression level of miR-378 but, rather, tissue-specific factors that must also play a role in the induction of tissue-specific fibrotic response.

In previous studies we and others have identified several members of the receptor-tyrosine kinase/Ras/ERK-signaling pathway as direct targets of miR-378 in cardiomyocytes (10, 12, 13). These included IGF1R, GRB-2, KSR-1, and ERK1. In the present study we found activation of downstream Ras effectors, Akt, S6K, and ERKs, and de-repression of IGF1R and GRB-2 in miR-378-depleted mouse hearts. In the receptor-tyrosine kinase/Ras/ERK signaling pathway GRB-2 is an adapter protein that is essential for the recruitment of SOS (son of sevenless) to the cell membrane and for the activation of the Ras-signaling cascade (40). KSR-1 is a scaffold protein required for binding of the Raf to Ras and for the activation of downstream MAPK cascade that includes MEK and ERK. Research has shown that PI3K- Akt as well as MAPK-ERK signaling cascades activate and also get activated by TGFβ signaling and that both c-Fos and c-Jun also serve as substrates for ERKs (see Ref. 41 for an excellent review). Therefore, by direct targeting of several members of the receptor-tyrosine kinase/Ras/ERK signaling pathway, miR-378 plays a significant role in the regulation of TGFβ synthesis and the activity of TGFβ signaling in the heart.

There are 10 miR-378 isomiR sequences that are described to originate from different genomic loci in the most recent available microRNA database and as illustrated in our previous pub-
miR-378 from its PGC1α mRNAs. An earlier study involving whole body knockout of miR-378 and miR-378-5p). These two miRNAs possess distinct seed sequences and, accordingly, target distinct sets of mRNAs. An earlier study involving whole body knockout of miR-378 from its PGC1α gene locus showed a metabolic role of miR-378 and miR-378* in the control of mitochondrial metabolism and maintenance of systemic energy homeostasis (14). These mice were found resistant to high fat diet-induced obesity, which correlated with direct targeting of carnitine-O-acetyltransferase (CRAT) by miR-378 and of mediator complex subunit 13 (MED-13) by miR-378*.

Intriguingly these targets were found to be de-repressed only in the liver tissues of the animals fed with high fat diet but not in the skeletal or cardiac tissues of miR-378 KO mice irrespective of normal chow or high fat diet, again suggesting a role of tissue-specific factors in miR-378-mediated targeting. Although the effects of genetic deletion of miR-378/miR-378* on cardiac hypertrophy and fibrotic markers were not analyzed in that study, the H&E staining of cardiac tissue of KO animals on high fat diet revealed no abnormalities in myofiber structure or organization. In our study short term treatment with antimiR, which was designed to target all forms of miR-378 but that spared miR-378*, we found evidence of cardiac hypertrophy and fibrosis suggesting that miR-378* acts as a pathological molecule in the absence of miR-378. Whether combined repression of mitogen-activated protein kinase pathway factors might contribute to the development of cardiac hypertrophy and fibrosis, a feature of deletion of miR-378a and miR-378* and/or the possibility that miR-378* acts as a pathological molecule in the absence of miR-378 remains to be addressed in future studies.

Based on our findings, we propose that miR-378 is a protective miRNA, and its potential role in ventricular remodeling during cardiac stress implicates that previously suggested targeting of miR-378 in metabolic disorders (14) should be revisited with caution as it could have deleterious consequences particularly in pre-existing myocardial diseases.

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Deficiency of Cardiomyocyte-specific MicroRNA-378 Contributes to the Development of Cardiac Fibrosis Involving a Transforming Growth Factor β (TGF β1)-dependent Paracrine Mechanism

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