Supplementary Material

MicroRNA-378 suppresses myocardial fibrosis through a paracrine mechanism at the early stage of cardiac hypertrophy following mechanical stress.

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MiR-378 is preferentially expressed in cardiomyocytes but not in cardiac fibroblasts

We performed a northern blot analysis of multiple mouse tissues and found that miR-378 is the most highly expressed in the heart and skeletal muscle (Sup. Figure 3A), which was also confirmed by real-time PCR (Sup. Figure 3B). By using real-time PCR we found that miR-378 was preferentially expressed in cardiomyocytes and had no expression in cardiac fibroblasts from neonatal rat hearts. We then examined the miR-378 expression in the cardiomyocytes and cardiac fibroblasts from both the adult wildtype mouse hearts and miR-378 knockout mouse hearts under sham and TAC conditions. In the sham-operated adult mice, we found that miR-378 had high expression in cardiomyocytes but had decreased expression in that of the miR-378 KO (+/-) mice, and we did not detect the expression of miR-378 in cardiac fibroblasts. In the TAC-operated mouse hearts, we surprisingly detected the expression of miR-378 in cardiac fibroblasts of wild-type mice, which may be originated from the caridomyocytes by paracrine mechanism under mechanical force (Sup. Figure 3C).

Supplemental Tables and Figures

Sup. Table 1

Echocardiographic assessment in mice in basal condition and treatments				
	Sham (n=9)	**2 week TAC (n=8)**	**Agomir 2 weeks TAC (n=7)**	**Antagomir 2 weeks TAC (n=8)**
BW (g)	22.8±0.9	22.2±0.4	21.9±0.5	22.7±0.8
HW/BW (mg/g)	4.49±0.10	6.76±0.39**	5.44±0.20*##	6.98±0.68**
HR (beat/min)	437±9	431±7	442±6	441±5
LVAWd (mm)	0.79±0.02	1.01±0.01**	0.88±0.03*##	1.15±0.02**#
LVAWs (mm)	1.02±0.02	1.29±0.03**	1.12±0.01*##	1.32±0.02**
LVPWd (mm)	0.75±0.01	0.98±0.02**	0.85±0.03*##	1.12±0.08**#
LVPWs (mm)	1.03±0.02	1.26±0.05**	1.10±0.03*##	1.29±0.08**
LVIDd (mm)	4.03±0.06	3.84±0.10**	3.94±0.06	4.02±0.05#
LVIDs (mm)	2.96±0.04	2.81±0.07	2.91±0.03	2.95±0.06
EF%	63.43±1.89	68.27±3.27	68.80±4.62	54.56±4.94#

Echocardiographic measurement in wildtype mice in basal condition and treatments, Related to Figure 1. Values are expressed as mean±SE. BW, body wight; HW, heart weight; HR, heart rate; LVAWd/s, left ventricle anterior wall thickness in diastole/systole; LVPWd/s, left ventricle posterior wall thickness in diastole/systole; LVIDd/s, left ventricular internal diameter in diastole/systole; EF%, ejection fraction. **, P< 0.01 vs. sham-operated hearts; *, P< 0.05 vs. sham-operated hearts; ##, P< 0.05 vs. TAC-operated hearts; #, P< 0.01 vs. TAC-operated hearts.

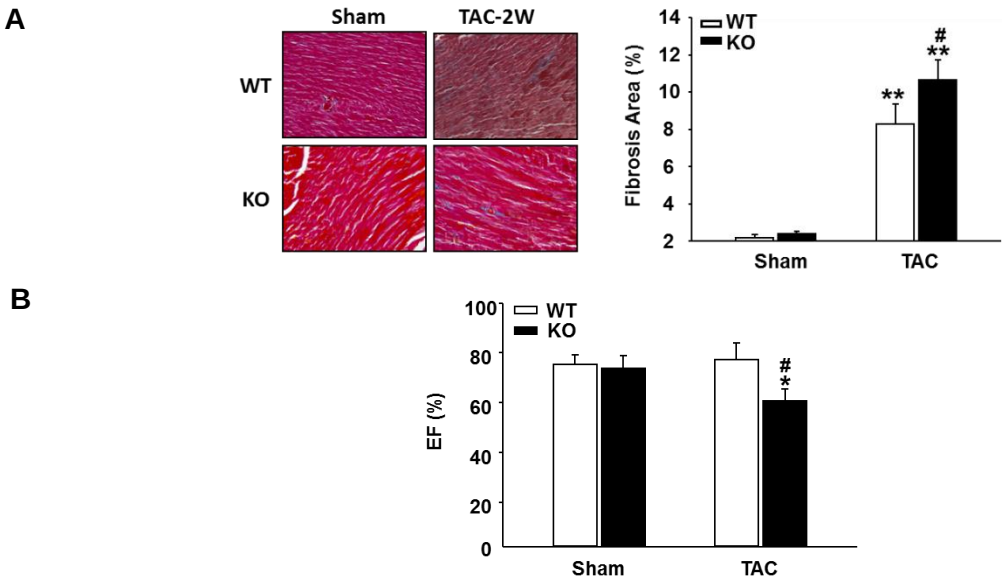
Sup. Table 2

Echocardiographic assessment in wildtype mice and miR-378 KO mice in basal condition and after 2 weeks TAC				
	Wildtype Sham (n=10)	**Wildtype 2 weeks TAC (n=7)**	**KO Sham (n=5)**	**KO 2 weeks TAC (n=8)**

BW (g)	22.5±0.3	23.7±0.4	22.9±0.7	22.1±0.9
HW/BW (mg/g)	5.06±0.24	6.69±0.31**	5.29±0.17	7.71±0.65**##
HR (beat/min)	427±5	457±7	422±4	438±6
LVAWd (mm)	0.77±0.04	0.95±0.02**	0.84±0.03	1.12±0.03**#
LVAWs (mm)	1.00±0.02	1.32±0.03**	1.12±0.03	1.52±0.08**
LVPWd (mm)	0.73±0.01	0.92±0.02**	0.73±0.02	1.10±0.04**#
LVPWs (mm)	1.05±0.02	1.28±0.06**	1.10±0.04	1.46±0.12**
LVIDd (mm)	3.95±0.06	3.35±0.04**	4.00±0.06	4.23±0.05##
LVIDs (mm)	2.96±0.04	2.02±0.03**	3.03±0.03	3.27±0.06#
EF%	62.63±1.89	71.08±3.14	58.29±3.67	51.65±3.14#

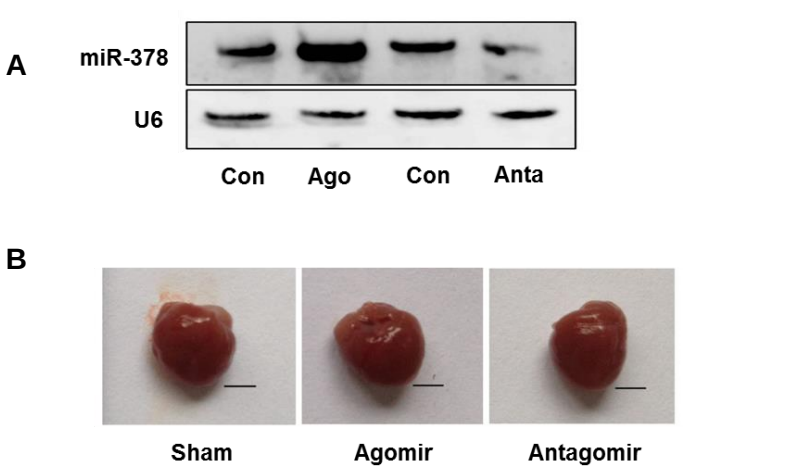
Echocardiographic measurement in wildtype mice and miR-378 KO (+/-) mice in sham condition and after 2 weeks TAC, Related to Figure 6. Values are expressed as mean±SE. BW, body wight; HW, heart weight; HR, heart rate; LVAWd/s, left ventricle anterior wall thickness in diastole/systole; LVPWd/s, left ventricle posterior wall thickness in diastole/systole; LVIDd/s, left ventricular internal diameter in diastole/systole; EF%, ejection fraction. **, P< 0.01 vs. sham-operated WT hearts; *, P< 0.05 vs. sham-operated WT hearts; ##, P< 0.05 vs. TAC-operated WT hearts; #, P< 0.01 vs. TAC-operated WT hearts.

Sup. Figure 1



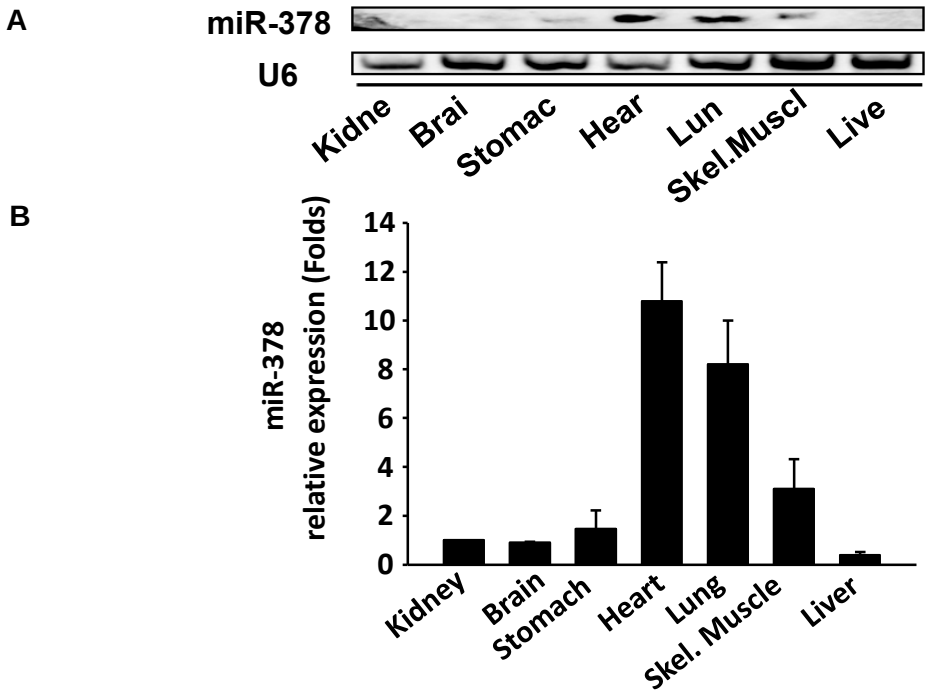
Cardiac remodeling and accompanying expression level of miR-378 of adult male wildtype (WT) and HSF1 knockout (KO) mice after 14 days TAC or sham operation, Related to Figure 1. A, Representative photographs of Masson-trichrome staining in LV wall. Quantitative analysis of fibrosis analyzed by calculated the radio of whole Masson-trichrome stained area to LV area. B, Ejection fraction value was measured by echocardiography. Values represent the mean ± SE. **, P< 0.01 vs. sham-operated WT hearts (n=3); *, P< 0.05 vs. sham-operated WT hearts (n=3); #, P< 0.05 vs. expression in TAC-operated WT hearts (n=3).

Sup. Figure 2



Intravenous injections of chemically modified miR-378 mimics (Agomir) or antisense RNA oligonucleotides (Antagomir), Related to Figure 1. A, After 14 days TAC, 15μg of total RNA was loaded on to detect miR-378 levels in the hearts by northern blot, U6 was used as a loading control. B, Representative photos of the hearts only treated by Agomir or Antagomir in sham operation (scale bar: 2 mm). C, The nucleotide sequences of miR-378 Agomir and Antagomir were shown. For Agomir, the antisense strand was cholesterol and phosphorthioate modified. The whole strand were 2′-OMe modified. For Antagomir, the single-stranded RNAs was cholesterol and phosphorthioate modified. The whole strand were 2′-OMe modified.

Sup. Figure 3



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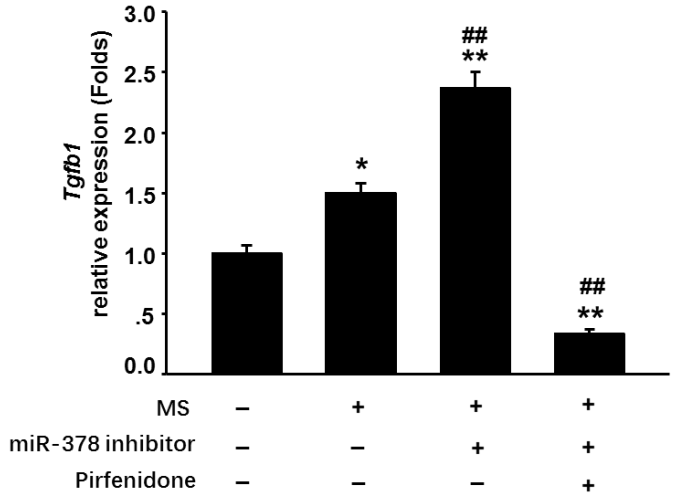
	CM(C_t)		CF(C_t)		
	MiR-378	U6	MiR-378	U6	Blank
New borne	27.19±0.22	20.40±0.12	39.75±0.20	18.85±0.25	39.25±0.12
Adults (WT-Sham)	22.69±0.06	21.45±0.09	38.75±0.14	23.72±0.08	38.25±0.06
Adults (WT-TAC)	28.67±0.24	25.70±0.02	28.67±0.12	25.74±0.02	37.49±0.08
Adults (KO-Sham)	24.79±0.06	21.67±0.08	39.73±0.03	22.43±0.03	39.32±0.11
Adults (KO-TAC)	30.37±0.22	22.84±0.02	36.85±0.09	20.82±0.12	38.94±0.21

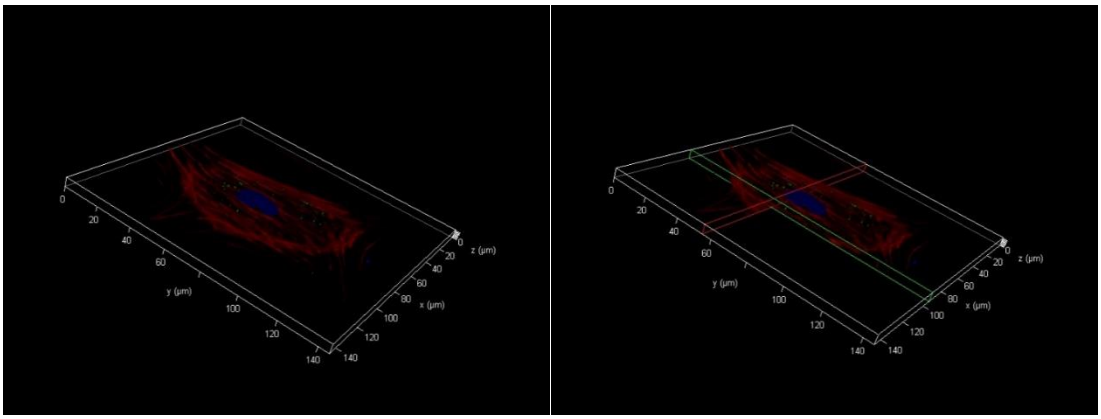
MiR-378 has a higher expression level in cardiomyocytes, Related to Figure 2 and Figure 6. A, Tissue distribution of miR-378 in mouse. Representative northern blot was performed on 15 μg of total RNA from various tissue samples. U6 was used as a loading control. B, Real-time PCR assay confirmed that miR-378 was enriched in heart tissues. Relative expression level was normalized by U6. C, The Ct values (mean±SE) (n=3) of miR-378 and U6 (as an internal control) by real-time PCR. The RNA samples are extracted from cardiomyocytes (CMs) and cardiac fibroblasts (CFs) of the neonatal or adult hearts. KO represents miR-378 knockout mice(+/-). All the adult mice underwent 2 weeks TAC. In the sham-operated adult mice, we found that miR-378 had high expression in cardiomyocytes but had decreased expression in that of the miR-378 KO (+/-) mice, and we did not detect the expression of miR-378 in cardiac fibroblasts. In the TAC-operated mouse hearts, we surprisingly detected the expression of miR-378 in cardiac fibroblasts of wild-type mice, which may be originated from the caridomyocytes by paracrine mechanism under mechanical force.

Sup. Figure 4



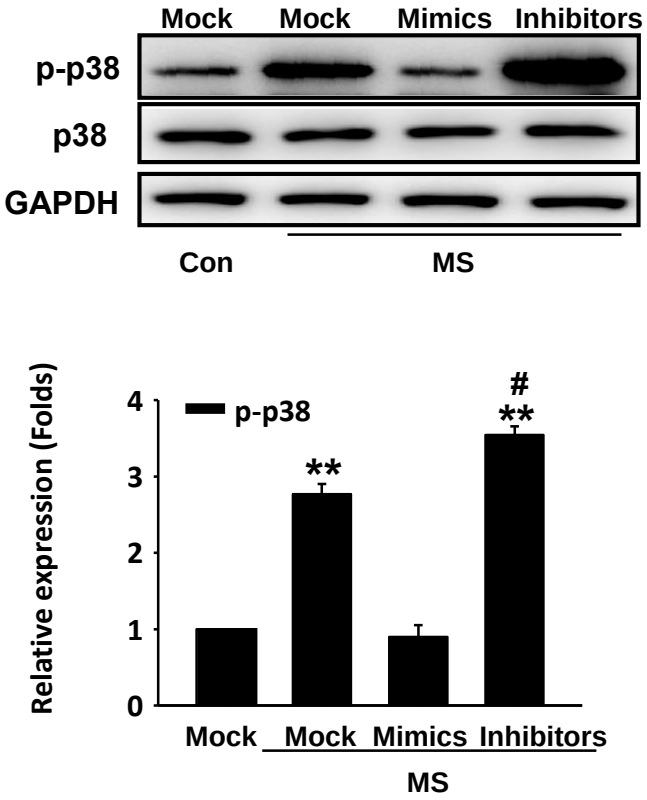
Quantitative real-time polymerase chain reaction analysis of gene *Tgfb1* (TGFβ1) in the cardiomyocytes, Related to Figure 3. Values represent the mean ± SE. **, P< 0.01 and *, P< 0.05 vs. control; ##, P< 0.05 vs. control with MS for 24hr.

Sup. Figure 5



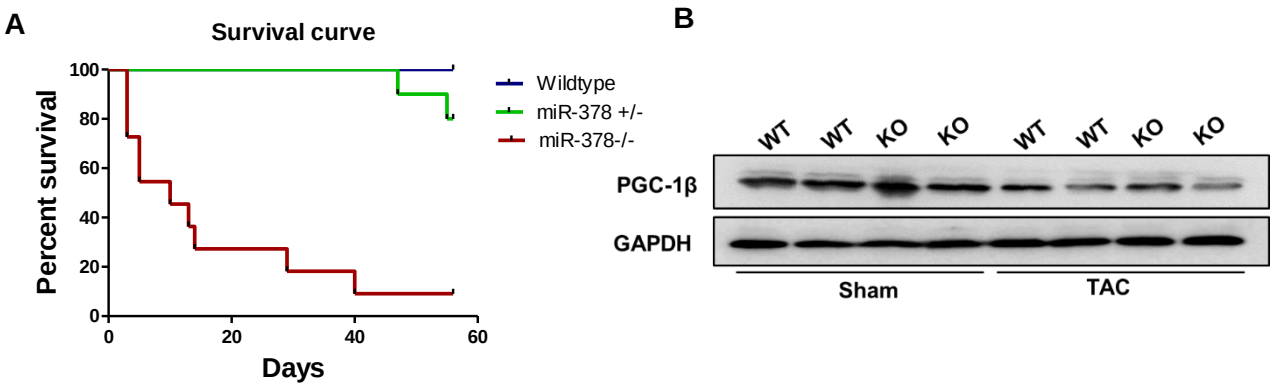
The 3D display of exosome uptake (green) into the CFs, Related to Figure 4.

Sup. Figure 6



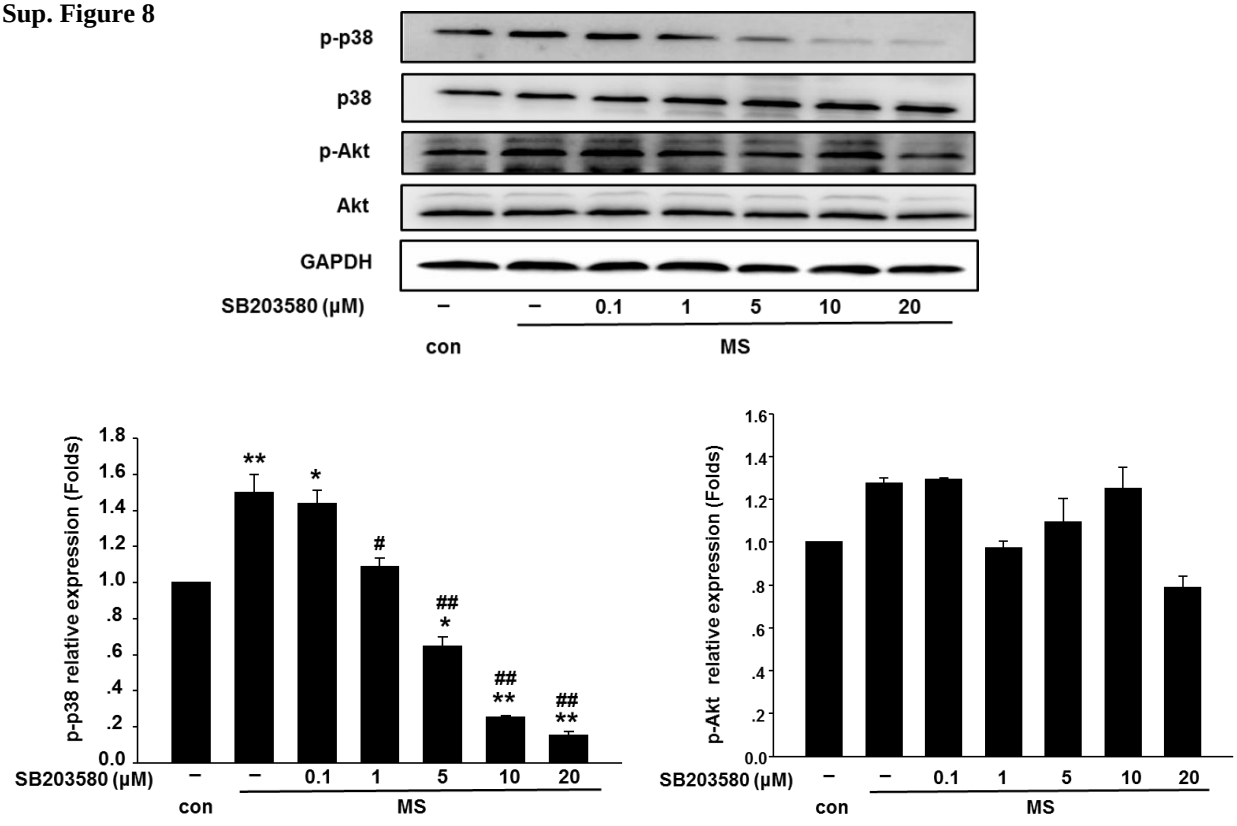
MiR-378 regulated the p38 MAPK in the cardiomyocytes, Related to Figure 2. Western blot and quantitative analysis of phosphorylated p38 MAPK and total p38 MAPK in cardiomyocytes. The cardiomyocytes were transfected with the chemically modified miR-378 mimics or the miR-378 inhibitors or the negative scramble control (mock), and at 48 h after transfection, the cells were mechanically stretched (MS) for 10 min or untreated. Values represent the mean ± SE from three independent experiments. **, P< 0.01 vs. mock CMs; #, P< 0.05 vs. mock CMs with MS.

Sup. Figure 7



Characterization of miRNA-378 knockout mice, Related to Figure 6. A, The survival curve for both miR-378 (+/-)mice and miR-378 (-/-)mice. No mortality was observed in wildtype littermates. B, Western blot of heart isolated from miR-378 KO (+/-) and WT mice using antibody against PGC-1β. GAPDH was used as a loading control.

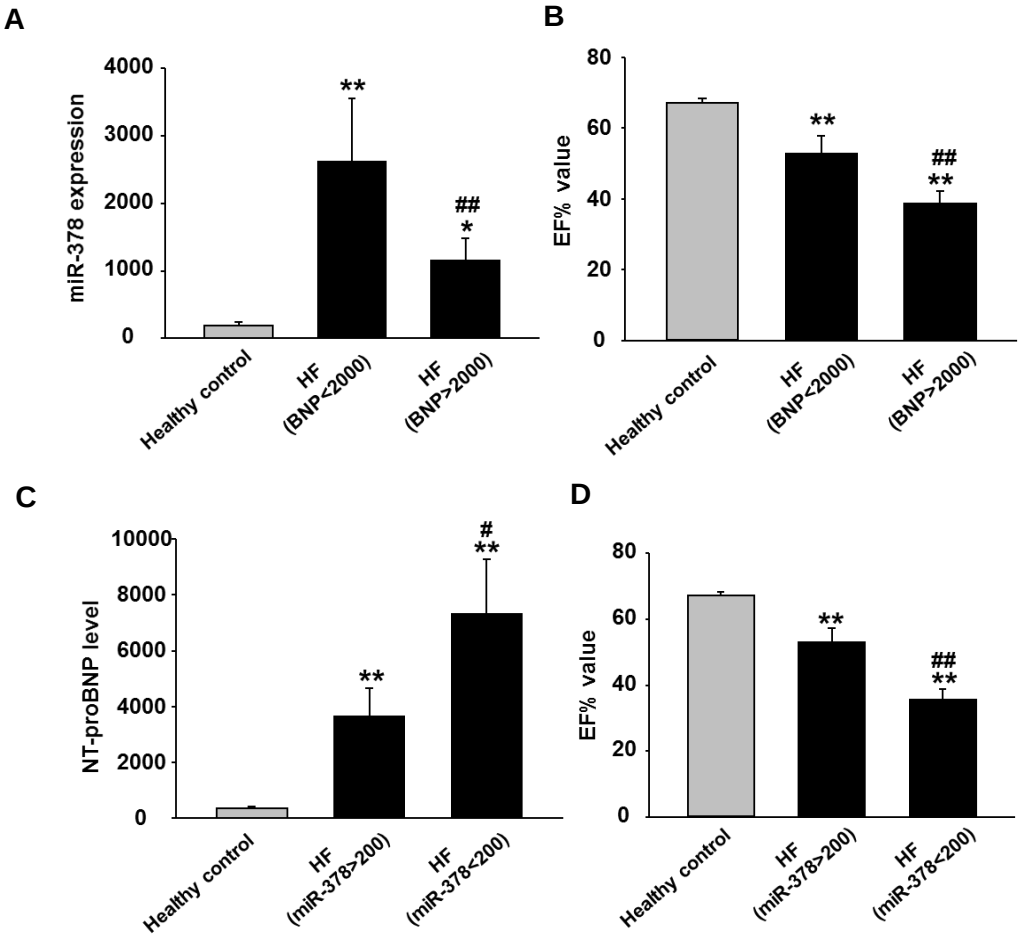
Sup. Figure 8



The dose effects of CFs treated with SB203580 on p38 MAPK and Akt activities, Related to Figure 5.

Western blotting and quantitative analysis of phosphorylated p38 MAPK, total p38, phosphorylated Akt and total Akt levels in the protein extracted from the CFs treated by different doses of SB203580 for 1 h prior to MS. Values represent the mean ± SE. **, P< 0.01 vs. control; *, P< 0.05 vs. control; ##, P< 0.01 vs. control MS; #, P< 0.05 vs. control MS. The results showed that 10 μM SB203580 did not affect Akt activation but 20 M SB203580 did.

Sup. Figure 9



Aberrant expression of circulating miR-378 in HF patients, Related to Figure 3. To depict the relationship between the expression trend of miR-378 in HF patients with different severity, we categorized HF patients according to their blood NT-proBNP level at the dividing point of 2000ng/L. (A) shows expression of miR-378 in serum and (B) shows EF% value in healthy control subjects(n=28), HF cases (NT-proBNP<2000) (n=12), and HF cases (NT-proBNP>2000) (n=15). Values represent the mean ± SE. **, P< 0.01 vs. healthy control; *, P< 0.05 vs. healthy control; ##, P< 0.01 vs. HF (BNP<2000). Then HF patients were categorized according their miR-378 levels in serum at the dividing point of 200 copies. (C) and (D) show blood NT-proBNP levels and EF% value in healthy controls (n=28), HF cases (miR-378>200) (n=11), and HF cases (miR-378<200) (n=16). Values represent the mean ± SE. **, P< 0.01 vs. healthy control; ##, P< 0.01 vs. HF (miR-378>200); #, P< 0.05 vs. HF (miR-378>200).

Supplemental Experimental Procedures

Clinical study cohort

Following ethical approval and informed consent, venous blood samples were collected from participants, including HF patients (n=27) and healthy controls (n=28). Experiments described were performed on samples obtained at Shanghai Zhongshan hospital at the time of diagnosis. All provided written consent and Ethic permission. In this study, we classified subjects as HF cases when they met the Framingham criteria for the diagnosis and NYHA class is above class I.

Analysis of miR-378 gene expression in human serum

Whole blood was separated into serum within 4h after blood was derived. For serum RNA isolation, equal volume of Trizol was used, and additional two more steps of phenol/chloroform purification since serum is full of proteins. The RNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The expression level of miR-378 was analyzed with a Taqman-based real-time quantitative PCR (RT-qPCR) using individual miRNA-specific primers and probes according to the manufacturer's protocol (Applied Biosystems). The first-strand miRNA- cDNA PCR template was reverse transcribed from 100 ng of total RNA using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). Synthetic standard mature miR-378 were also included in each batch of reactions. PCR reactions were carried out in final volumes of 10 μl using an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems). Reaction programs were performed according to the instruction, reactions were initiated with a 10-minute incubation at 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. We quantified the mature miR-378 through standard curve. Standard curves were generated for miR-378 assay using a dilution series of known input amounts of synthetic oligonucleotide. Raw PCR data (Ct values) were transformed into miR-378 copy numbers per RT reaction by SDS (Applied Biosystems) through extrapolation to the miR-378 standard curve (copies/ng).

Mouse TAC models

All animal procedures were approved by the Animal Care and Use Committee of Fudan University, and in compliance with guidelines for the Care and Use of Laboratory Animals published by the National Academy Press (NIH Publication No. 85-23, revised 1996). Adult mice were anesthetized via isoflurane inhalation and transferred to a heat-controlled pad to maintain a constant body temperature. Strong pressure overload was imposed on the heart of mice by TAC using a 27-gauge needle as described previously.(Zou et al., 2011) MiR-378 agomirs and miR-378 antagomirs were synthesized as described.(Krutzfeldt et al., 2005; van Solingen et al., 2009) Administration of intravenous injections started at the first day after TAC on three consecutive days (each 80mg/kg body weight). Transthoracic echocardiographic was performed with the use of Vevo770 echocardiograph (VisualSonics, Canada). Two-dimensional M-mode echocardiographic examination were performed using a 30MHz linear transducer at a stable heart rate of 500 beats/min. Haemodynamic measurements were performed by inserting a micronanometer catheter (Millar 1.4F, Millar Instruments) from the right common carotid artery. The transducer was connected to Power Laboratory system (AD Instruments) and blood pressure (BP) was measured. Animals in sham groups without elevation of BP (100~120 mmHg) and in TAC groups with elevation of BP by 40~50 mmHg were enclosed. Mice were sacrificed immediately after measurements and hearts were removed and weighed, quickly frozen in liquid nitrogen, and stored at -80℃.

Knockout mouse generation

Single-guide RNA (sgRNA) was designed by an online CRISPR Design Tool (http://tools.genome-engineering.org). To generate the sgRNA expression construct, vector pUC57-sgRNA (51132; Addgene, Cambridge, MA) was digested with BsaI, and the linearized vector was gel-purified. Synthesized oligonucleotides for targeting sites (
Target A: GG CTCAGAGCTGAGCGGGAA TGG; Target B: GG TGCTGACGGAGAAGCCAG AGG) were annealed and ligated to the linearized vector. The sgRNA expression plasmid was amplified by PCR with primers spanning T7 promoter and sgRNA regions. The T7-sgRNA PCR product was gel-purified and used as the template for transcription *in vitro*. A Cas9 expression plasmid (44758; Addgene) was linearized with PmeI and was used as the template for transcription *in vitro* using a T7 Ultra kit (AM1345; Thermo Scientific, Waltham, MA). Cas9 mRNA was purified using an RNeasy mini kit (74104; Qiagen, Stanford, VA) following manufacturer instructions. sgRNA was purified using a miRNeasy micro kit (217084; Qiagen). Cas9 RNA and sgRNA were injected into the cytoplasm of the mouse fertilized eggs with well-recognized pronuclei in M2 Medium (Sigma-Aldrich). The qualified embryos were transplanted into the fallopian tubes of the surrogate mice which then gave birth to the F0 generation mice. The F0 generation mice was examined by the genotyping. A pair of primers, Mir-378-check-F (5'- GATTGCCTGGAGTCGTGTCC-3′) and Mir-378-check-R (5'-TAGCCACCAAAGACAAGAAGAACTC -3′), was used to amplify the DNA fragment containing the CRISPR/Cas9 target site. PCR products were sequenced directly to allow identification of editing events. The wild-type (WT) allele yielded an amplicon of 890bp, whereas the mutant allele yielded an amplicon of 583bp. The F0 founders were mated with wildtype mice to obtain positive F1-generation. Thereby knockout mice model was established and bred.

Cell isolation and culture

Cells were grown in low glucose Dullbecco modified Eagle medium (DMEM) containing 10% FBS (Gibico) and antibiotics (penicillin and streptomycin). CFs were detached with 0.1% trypsin (Gibico) for passaging and 2 to 3 passages were used to study. Mechanic stretching of cells was conducted as described previously(Komuro et al., 1990). In brief, CMs were plated at a field density of 4×10^5 cells/ml on or silicone rubber culture dishes (20×40 mm) with 2 ml of culture medium. The culture medium was changed to serum free 12h before stretching. CMs were stretched by 20% with steel-made equipment. Similarly, CFs were plated at 3×10^5 cells/ml and stretched as CMs. Adult mouse cardiac fibroblasts were isolated by differential adhesion from the single-cell suspensions of the hearts. The single-cell suspensions were obtained using the gentle MACS^{TM} Dissociator (MACS).

Quantitation of mRNA and miRNA Expression

Total RNA was isolated from cells or mouse cardiac tissues with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Isolation of extracellular miRNAs in serum and cultured cell supernatant was performed using the miRcute miRNA Isolation kit (Tiangen).Quantification of miRNAs was performed with the TaqMan MicroRNA Assay Kit in accordance with the manufacturer's instructions (Applied Biosystems). For calculating relative fold change values, the C_T values were normalized to U6 miRNA as internal control. Analysis was done on Applied Biosystems 7500 Real-Time PCR instrument. For quantities of mRNA analysis, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA was used for real-time PCR with the SYBR® Premix EX Taq^{TM} (Takara). The reactions were incubated in a 96-well plate at 95℃for 2 min, followed by 40 cycles of 95℃ for 15 sec and 60℃for 1 min on an iCycler

IQ™5(Bio-Rad). All reactions were run in triplicate. Data were normalized to an endogenous control GAPDH.

Northern blot analysis

For northern blot analyses, 15 μg of total RNA were separated on a denaturing 15% polyacrylamide gel and transferred to NYTRAN®N membranes (Whatman) by a semi-dry electroblotting apparatus. Prehybridization was carried out for 40 min at 42℃ using ULTRAhyb-Oligo Hybridization Buffer (Ambion). Hybridization was carried out in the presence of 5'end-labeled LNA probe with biotin for miR-378 for 4hr. A probe U6, a ubiquitously expressed miRNA, was used as a loading control. After washing, the blots were exposed to visualized on LAS-3000 Imaging System (Fuji)

Histological analysis

Heart tissues were fixed in 10% formalin and embedded in paraffin or frozen in cryomolds, sectioned at 4μm thickness and stained with hematoxylin and eosin (H-E), Masson trichrome and antibodies against collagen I(Abcam), collagen III(Abcam) and α-SMA(Abcam). For measurement, five random high-power fields from each section were chosen and quantified in a blinded manner. The extent of fibrosis was measured in 5 sections from each heart.

Transfection of miRNA mimics and inhibitors.

CMs or CFs were switched into serum-free medium for 24hr prior to tansfection. The siPORT™ NeoFX™ Transfection Agent (Ambion) was used to transfect cells with mirVana™ miR-378 Mimics (35 nM) or mirVana™ miR-378 Inhibitors (50 nM) for 48 hr as per the manufacturer's instructions. MirVana™ miRNA inhibitor/mimic negative controls were used as mock.

EdU Incorporation Assay

Cells were exposed to 50μM of 5-ethynyl-29-deoxyuridine (EdU, RiboBio) for 2 hr at 37℃, and were fixed in 4% PFA for 30min at room temperature. After permeabilization with 0.5% TritonX-100, the cells were reacted with 1× Apollo reaction cocktail (RiboBio) for 30 min. Subsequently, the DNA contents of the cells were stained with Hoechst 33342 for 30 min and visualized under a laser scanning confocal microscopy (Leica, TCS Sp5).

Extracellular vesicles extraction from conditioned medium

Extracellular vesicles were purified from the conditioned medium of cardiomyocytes cultured in FBS free Opti-MEM® by the product Total Exosome Isolation (from cell culture media) (Life Technologies). Briefly, the supernatant was centrifuged at 2000 × g for 30 minutes to remove cells and debris. The 0.5 volumes of the Total Exosome Isolation (from cell culture media) reagent were added in the required volume of cell-free culture media. After mixed thoroughly, the culture media/reagent mixture was incubate at 4°C overnight. Then the samples were centrifuged at 10,000 × g for 1 hour at 4°C. Extracellular vesicles were contained in the pellet at the bottom of the tube and resuspend in 1X PBS. After extracellular vesicles were isolated, total RNA and protein were purified using the Total Exosome RNA and Protein Isolation Kit (Life Technologies).

Western blot analysis

Tissue or cells were lysed with 1× Cell lysis buffer (Cell Signaling) and protein isolation was done according to manufacturer's instructions. A measure 20–40 μg of protein was loaded on SDS–polyacrylamide gel electrophoresis gel for separation. Proteins were transferred to Immobilon-P membranes (Millipore) and incubated with first antibodies: MMP9(1:1,000; Abcam, ab38898), p-p38 (1:1,000; Cell Signaling, #9211), p-Smad2 (1:1,000; Cell Signaling, #3108), p-Smad3 (1:1,000; Cell Signaling, #9520),TGF beta1(1:1,000; Abcam, ab64715), GAPDH(1:10,000; Abcam, ab8245). HRP-

conjugated secondary antibodies were used for detection bands. Quantification of Western blots was performed by densitometry using the LAS-3000 Imaging System (Fuji) and NIH Image J software.

Supplemental References

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