Online Supplemental Material

Activation of dsRNA-dependent protein kinase R by microRNA-378 sustains metabolic inflammation in hepatic insulin resistance

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Online Supplemental Material

Cell culture, transfection and treatments: The mouse hepatocyte cell line AML12, rat hepatoma cell line McA-RH7777 (McA) and COS-7 cells were obtained from America Type Culture Collection (ATCC, Manassas, VI) and maintained in DMEM: F12 (1:1) or DMEM containing 10% FBS, 4.5g/L glucose and 0.5% penicillin/streptomycin, respectively. Cells were grown in T75 flasks at 37°C, 5% CO_2. For transfection, the cells were seeded onto 6-well plates (0.5×10^6) and maintained in appropriate media. When the cells reached 60~70% confluence, cells were transfected with 2.0μg of plasmid encompassing miR-378a (Vigene Biosciences, Rockville, MD) or scramble miRNA (10nM) (YM00479902-ADA, Qiagen, Hilden) as control by Lipofectamine 3000 for 48 or 60 hours. For 2-Aminopurine (2-AP) treatment, cells were transfected with 1.0 μg of plasmid encompassing miR-378a (Vigene Biosciences, Rockville, MD) or scramble miRNA (10nM) as control by Lipofectamine 3000 for 24 hours followed by treated with 2-AP (0.5mM, Sigma), or vehicle [PBS: Glacial acetic acid (200:1)] for additional 24 hours in DMEM without FBS (1). Total RNAs or whole cell lysates were collected at the end point of each treatment.

Seahorse (XF24) assay: McA cells were seeded at 1×10^4 cell/well in complete cell culture media 8 hours prior to transfection. Media were then replaced with DMEM (4.5g/L glucose) without FBS and antibiotics and transfected with 0.1μg of plasmid GFP (mock) or plasmid miR-378a using LIPO2000.

At 12 hours after transfection, cells were treated with WY1,4643 (30μM) or vehicle (DMSO), and incubated at zero CO_2 incubator for 24 hours. Then, cells were washed with XF Base media (1ml) twice, incubated with 1μM Oligomycin (Oligo), 1μM FCCP and 0.5μM Rotenone (Rot) at zero CO_2 incubator for 1 hour and subjected to Seahorse (XF24) analysis. Glucose, L-glutamine, and pyruvate were added to XF Base media to serve as substrates according to the manufacturer's instructions.

Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (IPITT): IPGTTs were performed as described in (2). Briefly, mice were fasted for 12 hours and baseline blood glucose was determined. Mice then received a dose of glucose (2g/kg) via IP injection and blood glucose levels were determined via tail vein bleeding at 15, 30, 60, 90 and 120 min using a handheld glucometer (Contour/Next, Bayer). For IPITT, the mice were fasted for 4h early in the morning. The baseline blood glucose levels were determined before insulin treatment. Mice were given a dose of insulin (0.5 U/kg) (Eli Lilly, Indianapolis, IN) via IP injection. And blood glucose levels were measured at 15, 30, 45, 60 and 90 min after insulin treatment.

PKR-miRNA Immunoprecipitation: McA cells were transfected with plasmid GFP (mock) or plasmid miR-378a (2μg) for 60 hours. Cells were then washed with ice-cold PBS and subjected to crosslinking under 1500 μJ × 100/cm^2 Ultraviolet (UV) prior to collection using liquid nitrogen snap-frozen and lysis with ice-cold sonication. Protein G beads (Promega: G747A) were washed twice with lysis buffer and incubated with an anti-PKR antibody (1:50) at room temperature for 60 min. The antibody-conjugated beads were then incubated with cell lysates and rotated at 4°C for 4 hours followed by washed three times with high-salt buffer and lysis buffer, respectively. The beads were then shaking-digested using Proteinase K (New England Biolabs: P8107S) at 37°C for 20 min to release the bound RNAs and proteins from beads. The bead-free immune-complexes were then subjected to standard TRizol/chloroform RNA extraction protocol. The mature bound-miR-378a in the immune-complexes was detected by TaqMan microRNA assay kits (Applied Biosystems, USA) according to the manufacturer's instructions. Briefly, RT-primers and PCR primers were provided by pre-designed TaqMan microRNA kits (Cat No. 4427975, assay ID 002243 for miR-378a-3p; Cat No. 4427975 assay ID 001232 for snoRNA202).

Reverse transcription was performed using the TaqMan microRNA reverse transcription kit (Applied Biosystems, Cat No 4366596). Each 15 µl reaction contained 10ng RNA template. Real time PCR was carried out using TaqMan universal PCR master mix II, no UNG (Applied Biosystems, Cat No 4440043) and normalized to snoRNA202.

Luciferase constructs and reporter assay: pMIR-Report miRNA expression vector system (AM5795) was used for constructing the luciferase expressing vector of mouse PPARα 3'-UTR. 1) WT PPARα 3'-UTR forward: 5'-agcttagctaggctttgaacataca**ctggact**gaaccctggattagagaacta-3; PPARα 3'-UTR reverse: 5'-ctagtagttctctaatccagggttc**agtccag**tgtatgttcaaagcctagcta-3'. 2) Luciferase construct with mutated targeted site was generated by replacing the targeted sequences of ctggact with cttgtcg. Mouse PPARα mut 3'-UTR forward: 5'-AGCTT agctaggctttgaacataca**cttgtcg**gaaccctggattagagaactA-3'; mouse PPARα mut 3-UTR reverse: 5'-CTAGTagttctctaatccagggttc**cgacaag**tgtatgttcaaagcctagctA-3'. The restriction enzymes used for cloning are SpeI and HindIII (Promega, WI, USA). For the luciferase reporter assays, COS-7 cells were co-transfected with miR-GFP (0.5µg)/pMIR-PPARα-3'UTR(0.5µg), miR-378a(0.5µg)/pMIR-PPARα-3'UTR(0.5µg), miR-378a(0.5µg)/pMIR-PPARα-3'UTR(0.5µg) mut plus Renilla (0.1µg). 48-hours post-transfection, miR-378a mediated luciferase activity was determined as the ratio of Firefly to Renilla using the commercial kit (Promega Dual-Luciferase Reporter Assay System, WI, USA).

Nanoparticle formulation: Nanoparticles loaded with anti-miR-378a were prepared using methoxy poly(ethylene glycol)-*block*-poly(2-methyl-2-carboxyl-propylenecarbonate-*graft*-dodecanol-graft-tetraethylene-pentamine) (mPEG-*b*-PCC-*g*-DC-*g*-TEPA) copolymer as described earlier (3). Briefly, NP carrying anti-miR-378a was prepared by hydrating the polymeric film in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing anti-miR-378a, vortex for 5 min and suspension was shaken for 25-30 min at room temperature to enable complex formation with miRNA. The mixture was then centrifuged at 5000 rpm for 5 min and filtered using 0.45 µm filter (Millipore). Nanoparticles carrying miRNAs was characterized by the particle size distribution and zeta potential

using a Malvern Zetasizer (NanoZS Series) and their morphology using a transmission electron microscope as described in (3).

Immunoblot analyses

Immunoblotting analysis was performed as previously described (2). The following antibodies were used: anti-PPARα (Abcam: ab8934), anti-Bip (Grp78) (Cell Signaling: 3183S), anti-phosphoserine-51 of eIF2α (Invitrogen: PA5-37800) ; anti-phosphor-JNK (Cell Signaling: 9255S), anti-phosphor-NFκB-p65 (Ser536) (Cell Signaling: 3033p), anti-HSP60 (Cell Signaling: 4870S), anti-phospho-IRS-1-Ser307 (Cell Signaling: 2381S), anti-phosphor-AKT (Cell Signaling: 9271S), anti-PKR and anti-phosphor-PKR Thr446 (Abcam: ab32036) polyclone antibodies. All antibodies were used at a final concentration of 0.1–1µg/ml. After incubation with the appropriate horseradish peroxidase-conjugated anti-mouse (GE Healthcare: NA931V) and anti-rabbit (GE Healthcare: NA934V) IgG secondary antibodies (1:2000 dilution), signals were detected using enhanced chemiluminescence (Pierce, Rockford IL).

miRNA extraction, miRNA sequencing profiling and data analysis

Total RNAs, including miRNAs, were isolated from the chow-fed or fructose-fed mouse livers using miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. miRNA sequencing profiling and data analysis conducted as in (4). Briefly, Illumina sequencing was performed by the Microarray Core Facility at the UT South Western Medical Center (USA) using Illimina HiSeq 2500 sequencer. Libraries were prepared using Illumina TruSeq Small RNA Sample preparation kit. Six replicates were sequenced for each treatment. Sequencing data were further confirmed by a TaqMan microRNA probe specific for miR-378a and normalized to snoRNA202 according to the manufacturer's instructions.

RNA isolation, reverse transcription and qRT-PCR

Total RNA was isolated from tissue using TRIzol (Life Technologies, Grand Island, NY). RNA integrity was confirmed using a NanoDrop 2000 (Wilmington, DE). First strand cDNA was synthesized with oligo (dT) and random primers using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies). Quantification of gene expression was performed by SYBR Green qPCR on a Roche LightCycler 480 Instrument II. Relative induction of gene mRNA expression was calculated

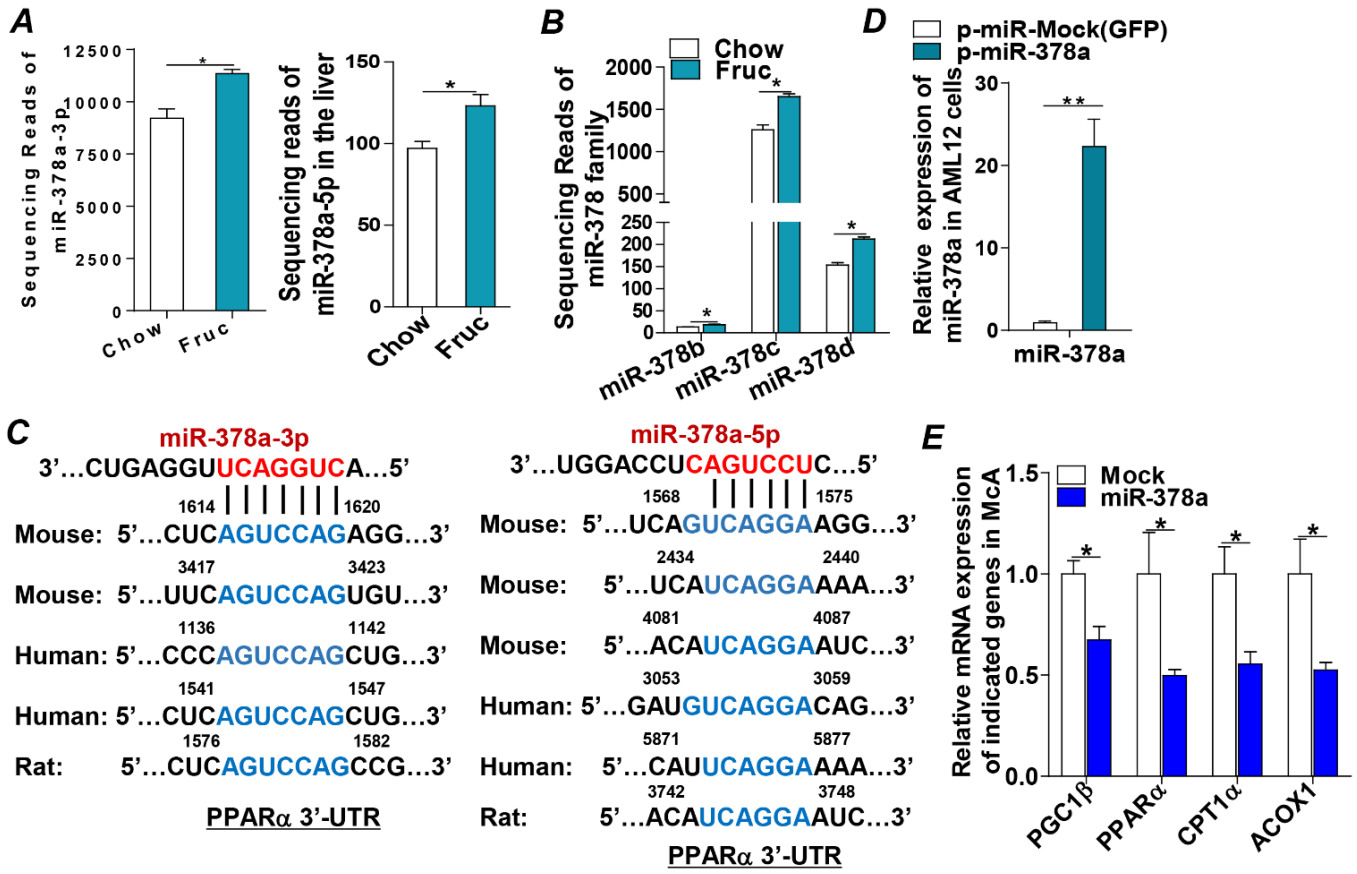
using the expression of 18s rRNA for normalization. Sequences of the primers used in this study are listed below.

Supplementary Table 1. Oligonucleotide sequences of primers for Real Time PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
IL-1β	TGGGCTGGACTGTTTCTA	ATCAGAGGCAAGGAGGAA
IL-6	AAGACAAAGCCAGAGTCCTTC	GTCCTTAGCCACTCCTTCTG
NFκB	CCCTGCGTTGGATTTCGT	AGTGGAGGCATGTTCGGTAG
TNFα	TCTCATTCCTGCTTGTGG	ACTTGGTGGTTTGCTACG
PPARα	CAACGGCGTCGAAGACAAA	TGACGGTCTCCACGGACAT
CPT1α	ACTCCGCTCGCTCATTCCG	CACACCCACCACCACGATAA
ACOX1	CTATGGGATCAGCCAGAAAGG	AGTCAAAGGCATCCACCAAAG
PGC1β	CTCTGACACGCAGGGTGG	GAAGAGCTCGGAGTCATCGG
ACADVL	CTCTATGGCACAAAGGCCCA	GGCTACATCGGATCCACTCG
MCAD	TTCGAAGACGTCAGAGTGCC	AGGGCATACTTCGTGGCTTC

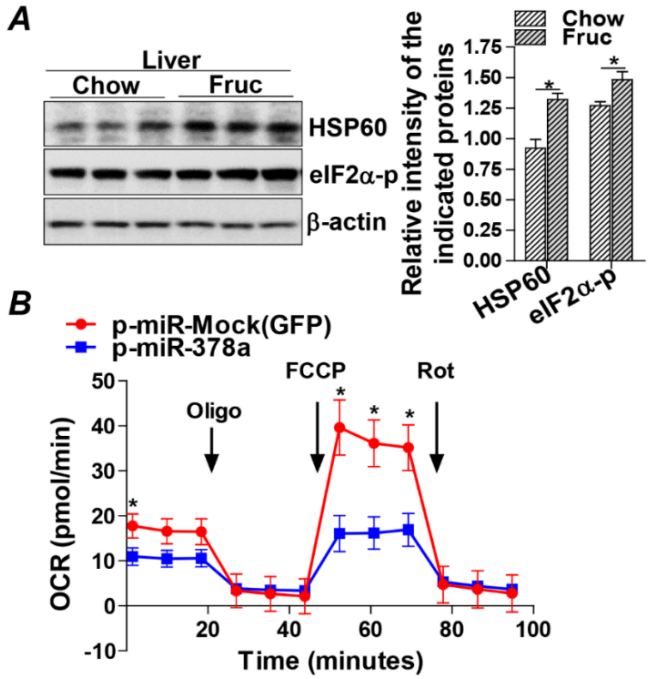
Supplementary Figures and Figure Legends

Supplementary Figure 1



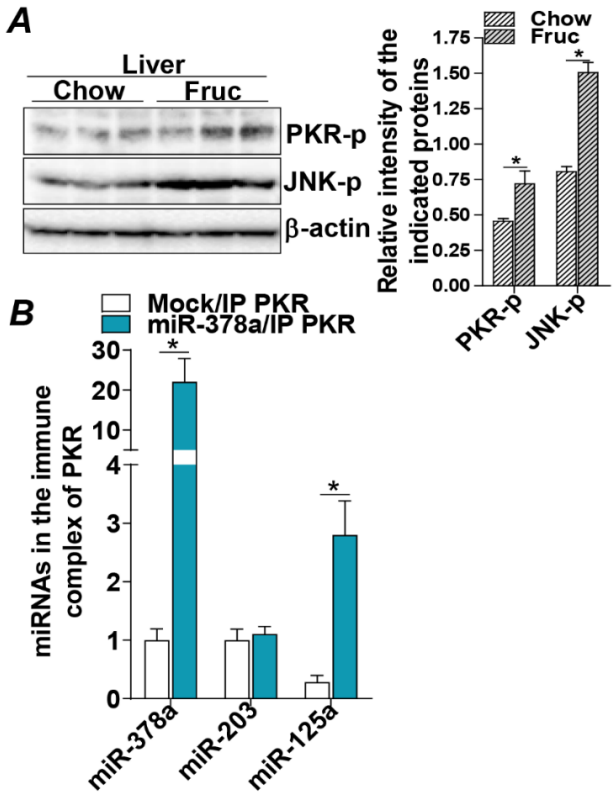
Supplementary Fig. 1. C57BL/6J mice (n=6/group) were subjected to chow or fructose fed for 4 weeks. Total miRNAs were extracted from the liver tissues and subjected to small RNA deep sequencing. (A) Sequencing counts of miR-378a-3p and -5p. (B) Sequencing counts of miR-378b, -c, and -d. (C) miR-378a response elements within the 3'-UTR of human, rat and mouse PPARα predicted by TargetScan. (D) AML12 cells were transfected with miR-378a or a mock vector (GFP) for 48 h followed by total RNA extraction. Expression of miR-378a was analyzed by a Taqman microRNA probe specific for miR-378a and normalized to snoRNA202. (E) McA cells were transfected with miR-378a or a mock vector (GFP) for 48 h. mRNAs of the indicated genes were detected by qRT-PCR. For cell experiments, two independent experiments were performed in triplicate. Results represent the means with SD. The two-tailed Student's t-test was used for statistical analyses of two-group comparisons. *P < 0.05 and **P<0.01 versus controls.

Supplementary Figure 2



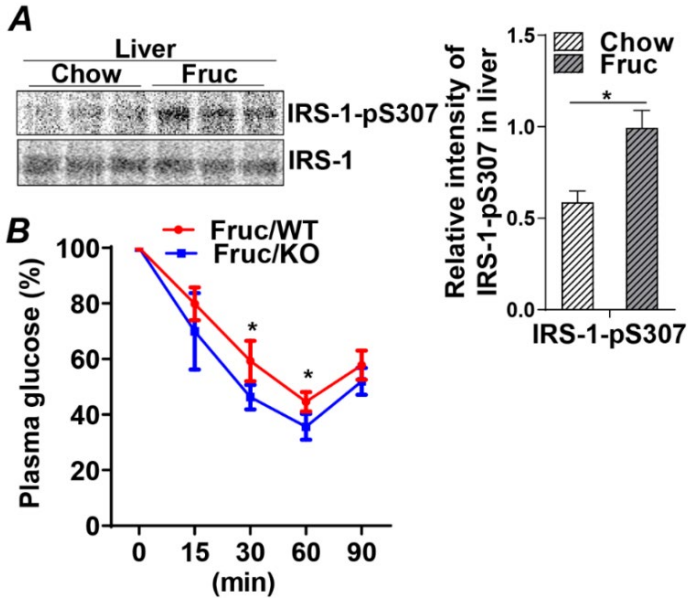
Supplementary Fig. 2. (A) Mitochondrial and ER stress markers HSP60 and eIF2α phosphorylation (eIF2α-p) determined by immunoblotting analysis in the livers of 4-week chow-fed or fructose-fed mice (C57BL/6J, n=6/group). (B) Oxygen consumption rate (OCR) was determined in McA cells transfected with miR-378a or mock (GFP) for 36 hours using Seahorse (FX24) analysis in the presence of 1µM Oligomycin (Oligo), 1µM FCCP and 0.5µM Rotenone (Rot). Results represent the means with SD from three independent measurements. The two-tailed Student's t-test was used for statistical analyses of two-group comparisons. *P < 0.05 versus controls.

Supplementary Figure 3



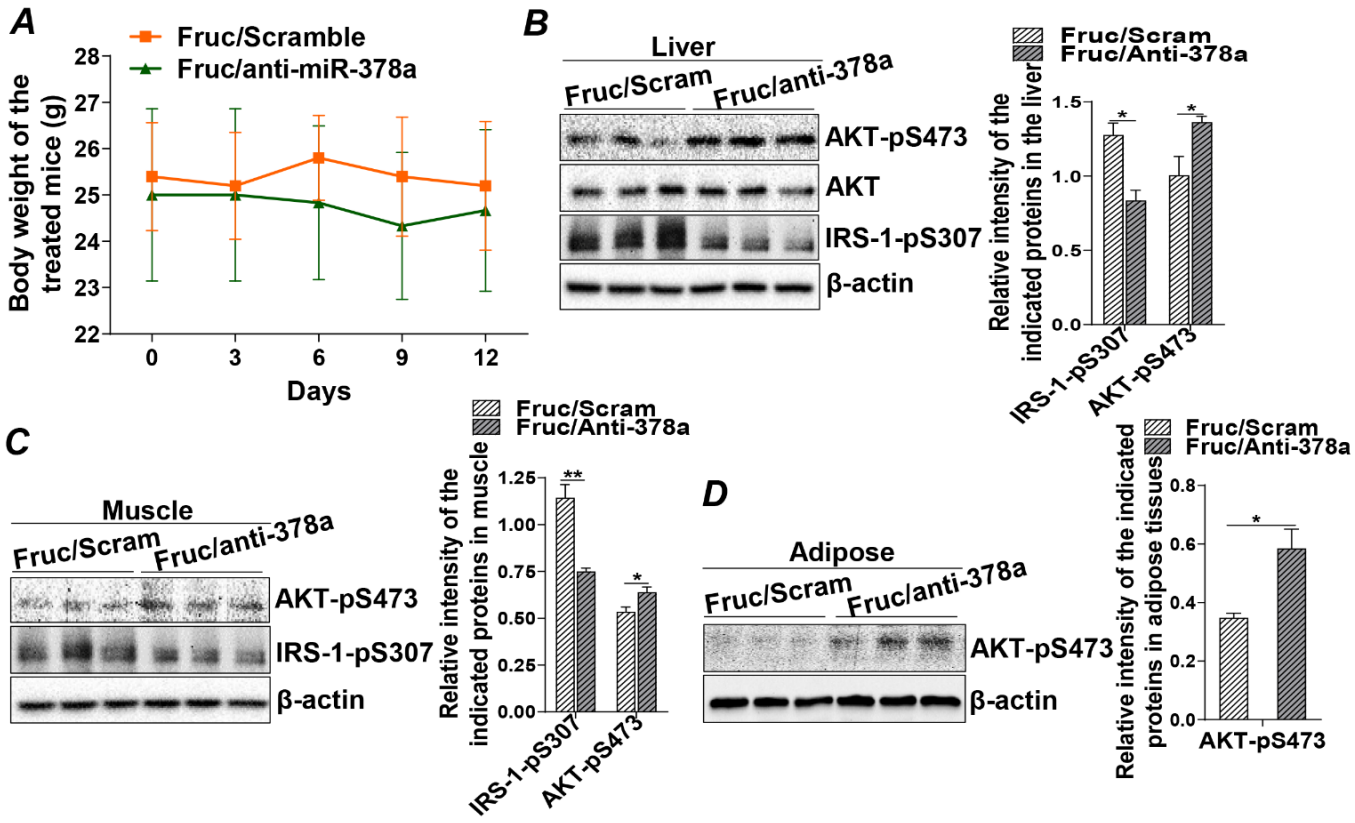
Supplementary Fig. 3. (A) Phosphorylation (active form) of PKR and JNK in the livers of 4-week chow-fed or fructose-fed mice (C57BL/6J, n=6/group) determined by immunoblotting analysis using phosphor-specific antibodies against PKR (PKR-p) and JNK (JNK-p). (B) Relative abundance of miR-378a, miR-125a and miR-203 was determined in the immune complex of PKR using miRNA specific primers and qRT-PCR followed by normalizing to the control sno202. Detailed procedure is provided in the online Supplemental Material of this manuscript. Results represent the means with SD. The two-tailed Student's t-test was used for statistical analyses of two-group comparisons. *P < 0.05 versus controls.

Supplementary Figure 4



Supporting Fig. 4. (A) Phosphorylation of IRS-1 at Ser307 in the livers of 4-week chow-fed or fructose-fed mice (C57BL/6J, n=6/group) determined by immunoblotting analysis using phosphor-specific antibodies. (B) Plasma glucose from an insulin tolerance test of the fructose-fed WT and miR-378a-KO mice (n=5/group). Results represent the means with SD. The two-tailed Student's t-test was used for statistical analyses of two-group comparisons. *P < 0.05 versus controls.

Supplementary Figure 5



Supporting Fig. 5. Mice (C57BL/6J) were subjected to a high fructose diet for 4 weeks. At week 3 of the feeding trial, mice were randomly divided into two groups (n=5/group) and subjected to treatment with a dose of nanoparticles either carrying the anti-miR-378a (3mg/kg) or scramble miRNA as control (3mg/kg) every three days through tail vein injection. Totally, four doses of nanoparticles were given to the mice by the end of the feeding trial. Tissues collected from the mice were subjected to the following analysis. (A) Body weights of the mice treated with anti-miR-378a or scramble miRNA. No significant changes were observed. (B-C) Phosphorylation of IRS-1 at Ser307 and AKT at Ser473 in the livers (B) and muscle (C) of the treated mice determined by immunoblotting analysis using phosphor-specific antibodies. (D) Phosphorylation of AKT at Ser473 in adipose tissue of the treated mice determined by immunoblotting analysis using phosphor-specific antibody. The two-tailed Student's t-test was used for statistical analyses of two-group comparisons. *P < 0.05 and **P<0.01 versus controls.

References

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