Supplementary Materials and Methods

Hepatocyte-specific miRNA Profiling

To identify miRNAs that were highly expressed in hepatocytes, we profiled global miRNAs in hepatocytes by comparing miRNA expression of livers between Dicer1^{fl/fl} (n=2) and Dicer1^{Δhep} mice (n=2) using Taqman Array MicroRNA Card (Applied Biosystems). These arrays were designed to investigate all miRNAs discovered in human, mouse and rat. Specifically, we isolated total RNA using the miRNeasy kit (Qiagen) from livers of Dicer1^{fl/fl} and Dicer1^{Δhep} mice\textsuperscript{1,2}, and the quality of total RNAs were determined using Bioanalyzer 2100. A total of 500 ng of RNA was used for miRNA-specific cDNA synthesis using Megaplex RT Primers and MicroRNA Reverse Transcription Kit (all Applied Biosystems). Taqman MicroRNA Array was run according to the manufacturer's protocol. Data analysis was performed using Viia 7 (Applied Biosystems) and Microsoft Access and the fold change was calculated using $2^{-△△Ct}$ method.\textsuperscript{3} Internal control was U6 nuclear small RNA and U6 was measured five times in each sample. miR-21 expression was further confirmed between Dicer1 knockout and wild-type mice using Taqman microRNA Assay from Invitrogen.

Fatty Acid Treatment of HepG2 Cells

HepG2 cells were seeded in a 4-well chamber slides using DMEM medium with 10% FBS and allowed to adhere overnight. To determine the inductive effect of miR-21 on lipogenesis, HepG2 cells were then treated with DMEM supplemented with 1% fatty acid free BSA and oleate (0.25 mM). Simultaneously, HepG2 cells cultured in the DMEM containing 0.25 mM oleate were transfected with miR-21 mimic (40 nM) or scrambled control (both Dharmacon) using Lipofectamine 2000. After 24 hours of transfection, Nile-Red Staining was used to
determine the intracellular lipid content in HepG2 cells. To investigate the inhibitory effect of miR-21 inhibitor on lipogenesis, HepG2 cells were then treated with DMEM supplemented with 1% fatty acid free BSA and oleate (0.5 mM). Simultaneously, HepG2 cells cultured with the DMEM containing 0.5 mM oleate were transfected with miR-21-ASO (40 nM) or miR-21-MM-ASO (control) (Both Exiqon). Lipid accumulation was determined by Nile-Red Staining followed by microfluorimeter detection or imaging.

*p53* expression vector (Plasmid #: 12091) and *p53* and *Hbp1* siRNA expression vectors were purchased from Addgene and Origene, respectively; and *HBPI* expression vector (pPM-hHBPI-His vector) was purchased from Applied Biological Materials (ABM) Inc. To determine whether the inhibitory effect of miR-21-ASO on lipogenesis is mediated by *HBPI*, HepG2 cells cultured in the DMEM containing 0.5 mM oleate were transfected with miR-21-ASO (40 nM in 4-well chamber slides), or miR-21-ASO combined with *HBPI* siRNA expression vector (200 ng/well in 4-well chamber slides). To determine whether *p53* is able to impair the ability of miR-21 mimic to induce lipogenesis, HepG2 cells cultured in the DMEM containing 0.25 mM oleate were transfected with miR-21 mimic (40 nM) or a combination of miR-21 mimic and *p53* expression vector (200 ng/well in 4-well chamber slides). To further determine the role of the interaction between miR-21 and *p53*, HepG2 cells cultured in the DMEM containing 0.5 mM oleate were transfected with miR-21-ASO (40 nM) or a combination of miR-21-ASO and *p53* siRNA (100 ng). Lipofectamine 2000 was used for miR-21 mimic and miR-21-ASO transfection. The cells were cultured for an additional 24 hours, after which lipid accumulation was determined by Nile Red Staining (Sigma-Aldrich).

*Lipid Accumulation Assay*
The lipid content in HepG2 cells was determined using Nile Red, a vital lipophilic dye (9-diethylamino-5H-benzo [alpha] phenoxazine-5-one) from Sigma-Aldrich, which has been shown to selectively stain intracellular lipid droplets. Monolayers were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 10 minutes. After washing, the cells were incubated for 20 minutes with Nile Red solution at a final concentration of 1mg/L in PBS at 37°C. After removal of chamber, the slides were mounted with Prolong® Gold anti-fade reagent with DAPI (Invitrogen) for visualization under fluorescence microscope.

**Reporter Vector Construction and Luciferase Assay**

To generate the luciferase reporter vectors, Hbp1 3’ UTR was amplified by PCR from mouse cDNA, and inserted into the pMiR-Reporter vector (Ambion), referred as to pMiR-Hbp1. Two bases of the binding site for miR-21 within the 3’UTR of Hbp1 were mutated using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) per the manufacture’s instruction, and referred as to pMiR-Hbp1-Mu. Twenty-four hours before transfection, 5×10^4 Hepa1,6 cells were plated per well in a 24-well plate. Then, 200 ng of pMiR-Hbp1 and miR-21 mimic or miR-21 inhibitor (20 nM or 40 nM) as well as 30 ng of β-gal plasmid pSV-β-Galactosidase Control Vector (Promega) were transfected using Lipofectamine 2000 (Invitrogen). Scrambled control (Dharmacon) or miR-21-MM-ASO (Exiqon) was used as the control for miR-21 mimic or miR-21-ASO, respectively.

*p53* promoter (1.5 kb) was amplified from mouse genomic DNA and was cloned into pGL3-basic vector (Promega), referred as to pGL3-p53. 24 hours before transfection, 5×10^4 cells Hepa 1,6 were plated per well in a 24-well plate. Then, 200 ng of pGL3-p53 with 100 ng or 200 ng HBPI expression vector (pPM-hHBPI-His vector) or mice HBPI siRNA expression vector (Origene) as well as 30 ng of β-gal plasmid pSV-β-Galactosidase Control Vector (Promega).
were transfected into Hepa1, 6 cells using Lipofectamine 2000 (Invitrogen). After 24 hours of transfection, luciferase and β-galactosidase assays were done using the Luciferase Assay System and Beta-Glo® Assay System (Promega). Luciferase activities were normalized to galactosidase activities; wells were transfected in triplicate; and each well was assayed in triplicate.

*Srebp1c* promoters (1.5 kb) were amplified from mouse genomic DNA and was cloned into pGL3-basic vector, referred as to pGL3-*Srebp1c*. 24 hours before transfection, 5×10⁴ Hepa 1,6 cells were plated per well in a 24-well plate. Then, 200 ng of pGL3-*Srebp1c* with 100 ng or 200 ng *p53* expression vector or *p53* siRNA expression vector (100 ng) and 30 ng of β-gal plasmid pSV-β-Galactosidase Control Vector (Promega) were transfected into Hepa1,6 cells using Lipofectamine 2000 (Invitrogen). After 24 hours, luciferase and β-galactosidase assays were performed using the Luciferase Assay System and Beta-Glo® Assay System (Promega). Luciferase activities were normalized to galactosidase activities; wells were transfected in triplicate; and each well was assayed in triplicate.

**miRNA Transfection and Gene Expression**

5×10⁴ of HepG2 cells were seeded in a 24-well plate and allowed to adhere overnight. To determine the effects of miR-21 overexpression and knockdown on gene expression, HepG2 cells cultured in the DMEM with 10% FBS were transfected with miR-21 mimic (40 nM) or inhibitor (40 nM) using Lipofectamine 2000. The equal concentration of scrambled control or miR-21-MM-ASO was used as control for miR-21 mimic or miR-21-ASO, respectively. 24 hours after transfection, cells were washed using cold PBS and the total RNA were isolated for gene expression analysis.

5×10⁴ of HepG2 cells were seeded in a 24-well plate and allowed to adhere overnight. To overexpress *HBP1* or *p53*, 200 ng of pPM-hHBP1-His vector (Applied Biological Materials
(ABM) Inc.,) or p53 expression vector (Addgene) was transfected into HepG2 cells using Lipofectamine 2000. The control HepG2 cells received empty pcDNA™3.1 vector (Life Technologies). 48 hours after transfection, cells were harvested for RNA isolation and gene expression analysis. To knock down p53 or HBP1, human p53 siRNA expression vector (100 ng) or HBP1 siRNA expression vector (100 ng) was introduced into HepG2 cells using Lipofectamine 2000. 48 hours after transfection, the HepG2 cells were collected for RNA isolation and gene expression analysis.

To study whether HBP1 mediates the inhibitory effect of miR-21-ASO on lipogenesis, 5×10^4 of HepG2 cells were seeded in a 24-well plate and allowed to adhere overnight. Then, the cells cultured in the DMEM containing 0.5 mM oleate were transfected with miR-21-ASO (40 nM), or a combination of miR-21-ASO with HBP1 siRNA expression vector (200 ng/well). After 48 h, the HepG2 cells were collected for lipid content determination and gene expression analysis. Lipid content in HepG2 cells were determined using Nile-Read staining and gene expression was measured using qRT-PCR.

**Histological analysis**

Liver samples were embedded in Tissue-Tek OCT embedding compound, and frozen on dry ice. 8 µm-thick sections were cut with a Leica CM3050 S cryostat, air-dried, and fixed in 10% formalin. After washing, sections were stained with an Oil-Red-O (Sigma-Aldrich)/60% isopropanol solution (Fisher Scientific). Briefly, sections were rinsed with 60% isopropanol and stained for 20 min with prepared Oil Red O solution (0.5% in isopropanol followed by dilution to 60% with distilled water and filtered). After rinses in 60% isopropanol and distilled water, slides were counterstained with hematoxylin for 4 min, rinsed with water, and mounted. Hematoxylin and Eosin Staining kit (Scytek laboratories, Inc.) was used in paraformaldehyde-
fixed, paraffin-embedded sections of liver according to manufacturer's protocol. Images were taken with Zeiss Axioplan 2 Upright Microscope.

**RNA Isolation and Quantitative Reverse Transcription-PCR (qRT-PCR)**

Total RNA was isolated with miRNeasy Mini Kit (Qiagen). To assess gene expression, 1 μg RNA was used for cDNA synthesis with Superscript III reverse transcription reagent (Invitrogen). PCR amplification was performed at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute in a 7900 real time-PCR system with SYBR green (Applied Biosystems). For each sample, we analyzed β-actin, GAPDH or 18S rRNA expression to normalize target gene expression. Primers for qRT-PCR were designed with Primer Express software (Applied Biosystems). Primers used for quantitative RT-PCR were listed in Supplementary Table 4.

To determine levels of miRNA expression, 10 ng RNA were used for miRNA-specific cDNA synthesis with the TaqMan MicroRNA Reverse Transcription Kit and Taqman MicroRNA Assays (all Applied Biosystems). PCR amplification was performed at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute in a 7900 real time-PCR system (Applied Biosystems). The small RNA Sno202 and RNU6 were used to normalize target miRNA expression. Relative changes in gene and miRNA expression were determined using the 2\(^{-\Delta\Delta Ct}\) method.\(^3\)

**MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) Assay**

Cell proliferation was determined using a MTT cell proliferation kit (Cell Biolabs, Inc.). 5\(\times\)10\(^3\) of HepG2 cells were seeded in each 96-well plate and allowed to adhere overnight. The cells were then transfected with scrambled control (40 nM), miR-21-ASO (40 nM), or miR-21
mimics (40 nM). After 48 hours culture, cells were used for MTT assay per the manufacturer’s instruction (Cell Biolabs, Inc). To determine whether HBP1 or p53 mediates the inhibitory effect of miR-21-ASO on cell proliferation, we transfected HepG2 cells with miR-21-ASO (40 nM) or a combination of miR-21-ASO and HBP1 siRNA expression vector (100 ng) or p53 siRNA expression vector (100 ng). After 48 hours culture, cells were used for MTT assay per the manufacturer’s instruction (Cell Biolabs, Inc). To determine whether p53 overexpression was able to antagonize the effect of miR-21 on proliferation, we transfected miR-21 mimics (20 nM) or a combination of miR-21 mimics (20 nM) and p53 expression vector (100 ng) in a 24-well plate. After 48 hours of transfection, cells were used for MTT assay per the manufacturer’s instruction (Cell Biolabs, Inc). HepG2 cells were transfected with lipofectamine 2000.

Soft Agar Colony Formation Assay

HepG2 cells (0.5 × 10⁶) in 35-mm plastic dishes were transfected with miR-21 mimics (40 nM) or inhibitors (40 nM). Two days after transfection, transfected cells were suspended with 8 ml of 0.4% top agar (Sigma-Aldrich) and 2×DMEM supplemented with 20% fetal bovine serum before being poured onto 6-cm tissue culture dishes coated with 3.5 ml of 0.7% bottom agar. Fourteen days later, three areas per plate were chosen randomly, the number of visible colonies was counted. To determine whether HBP1 and p53 mediate the inhibitory effects of miR-21-ASO on HepG2 cells colony formation, HepG2 cells were transfected with miR-21-ASO (40 nM) or a combination of miR-21-ASO (40 nM) and p53 siRNA expression vector (100 ng) or HBP1 siRNA expression vector (1 µg). After transfection for 48 hours, cells were suspended with 8 ml of 0.4% top agar (Sigma-Aldrich) and 2×DMEM supplemented with 20% fetal bovine serum before being poured onto 6-cm tissue culture dishes coated with 3.5 ml of 0.7% bottom agar.
Fourteen days later, three areas per plate were chosen randomly, the number of visible colonies was counted.

**Xenograft Tumor Assay**

HepG2 cells were placed in a 6-well plate 24 hours prior to transfection. HepG2 cells were transfected miR-21-ASO (40 nM), miR-21-MM-ASO (40 nM) or a combination of miR-21-ASO and HBP1 siRNA expression vector (1 µg) or p53 siRNA expression vector (400 ng). After 24 hours, 5×10^5 cells in 0.1 ml PBS were injected subcutaneously into the right flank of athymic nude mice (n=9) to establish a model of tumor-bearing mice. Tumor growth was observed every 3 days by measuring its diameter with Vernier calipers. Tumor weight was calculated by gram. Tumor volume (cm³) = d² × D/2, where d is the shortest and D is the longest diameter, respectively. Mice were sacrificed when the tumor size reached 1.5 cm in diameter. All protocols complied with, and all animals received humane care according to, the criteria outlined in the NIH “Guide for the Care and Use of Laboratory Animals.”

**Cell Cycle Analysis**

HepG2 cells were plated in a 6-well plate 24 hours before transfection. After 48 hours of transfection with miR-21 mimic (40 nM) or inhibitor (40 nM), the cells were detached from the plates by trypsin incubation, rinsed with PBS and fixed in 70% (v/v) ethanol. They were then re-suspended in PBS and incubated with RNase (100 µg/ml) and propidium iodide (60 µg/ml) (Sigma-Aldrich). Cells were analyzed using the FACSCalibur System (BD Biosciences), and the cell cycle phase was analyzed by using CellQuest software. The proliferation index (PI) was calculated as follows: PI = (S+G2/M)/G1. S, G2/M and G1 refer to the percentage of cells in S phase, G2/M phase and G1 phase, respectively. PI=(S+G2/M)/G1. To determine whether p53 or HBP1 mediates the inhibitory effect of miR-21-ASO on cell cycle progression, HepG2 cells
were transfected with miR-21-ASO (40 nM) or a combination of miR-21-ASO (40 nM) and HBP1 siRNA expression vector (1 µg) or p53 siRNA expression vector (100 ng). After 48 hours of transfection, the HepG2 cells were treated as above for cell cycle analysis.

**Human Cell Cycle RT² Profiler™ PCR Array**

We determined the effect of HBP1 on expression of genes controlling cell cycle using Human Cell Cycle RT² Profiler™ PCR Array (Qiagen). The Human Cell Cycle RT² Profiler™ PCR Array profiles the expression of 84 genes key to cell cycle regulation. Briefly, HepG2 cells were plated in a 6 well plate 24 hours prior to transfection. HepG2 cells were transfected with 1 µg of pPM-hHBP1-His vector (Applied Biological Materials (ABM) Inc.,) using Lipofectamine 2000. The control HepG2 cells were treated with empty pcDNA™3.1 vector (Life Technologies). 48 hours after transfection, cells were harvested for RNA extract. A total of 2 µg RNA was used to perform the reverse transcription using SuperScript® III Reverse Transcriptase (Invitrogen). Cell Cycle RT² Profiler™ PCR Array was run according to the manufacturer's protocol. Data analysis was performed using $2^{-\Delta\Delta Ct}$ method. Internal control is β-actin. p53 expression was further confirmed between HBP1 expressed vector treated cells and pcDNA3.1 vector treated cells using qRT-PCR.

**Plasma Lipid Analysis**

Blood was collected into tubes, containing 4 mM of EDTA, from cardiac puncture of C57Bl/6 mice after 4 or 8 weeks of HFD treatment. Plasma was separated by centrifugation (3000 x RPM for 20 min at 4 °C and triglyceride (mg/dl, Roche Diagnostics) was quantified enzymatically. Serum chemistry was carried out by the Pathology Laboratory of the University of Minnesota.
**Hepatic Lipid Analysis**

Mouse liver (100 mg) was placed in 1 ml chloroform/methanol (2:1) mixture and incubated on mice for 10 minutes before homogenization. Lipids were extracted from liver homogenates through room temperature orbital shaking (2 hours) followed by centrifugation (5000 RPM for 5 minutes). Supernatants were collected and washed with 0.4 ml chloroform/methanol (2:1) mixture by centrifugation at 5000 RPM for 20 minutes (room temperature). New supernatants were washed with 0.2 volume of 0.9% NaCl. After centrifuging for 5 minutes at 5000 RPM, supernatants were removed and lower-phase was dried at 42°C. Dried lipids were re-suspended in 2% Triton X-100. Liver triglycerides were quantified via a colorimetric assay using a triglyceride assay kit from Roche Diagnostics according to the manufacturer’s protocols.

**Western Blot Analysis**

Western blot was performed following standard procedures. HBP1 primary antibodies were purchased from Abcam; and binding was visualized using SuperSignal west femto maximum sensitivity substrate (Cat # 34095, Thermo Fisher Scientific Inc.).
References

