MicroRNA-206 prevents hepatosteatosis and hyperglycemia by facilitating insulin signaling and impairing lipogenesis

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Supplementary materials and methods

Identification of miR-206 targets

Identification of miR-206 target genes was conducted as previously described with minor revision (1). To identify genes with binding motifs for miR-206, we downloaded the target gene databases of miR-206 based on TargetScan (2), Pictar (3), and Starbase (4). Only hits from TargetScan or PicTar algorithm that were confirmed by Ago HITS-CLIP (high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP) from Argonaute protein complex) were selected. These three databases were compared using Microsoft Access 2000. We then carried out Gene Ontology analysis using PathwayStudio software (Elsevier) and compared with established and potential therapeutic targets for HCC(5-9) and obesity-related metabolic disorders (10), yielding 6 potential targets that have miR-206 binding motif (Supplementary Table 1). Our prediction from in silico algorithms showed that 3’ UTRs of Ptpn1 mRNA are 100% complementary to the miR-206 5’ seed region exhibiting the highest prediction scores and binding energy. In addition, the seed regions are conserved between the species of mouse, human and rat.

Reporter vector construction and luciferase Assay

To generate the luciferase reporter vectors, 3’ UTR of Ptpn1 were amplified by PCR from mouse cDNA, and inserted into the pMiR-Reporter vector (Ambion), referred as pMiR-Ptpn1. Two bases of the binding sites for miR-206 within the 3’UTR of Ptpn1 were mutated using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) per the manufacture’s instruction, and referred as pMiR-Mu-Ptpn1.
before transfection, 5×10^4 Hepa1,6 cells were plated per well in a 24-well plate. Then, 200 ng of the luciferase reporter vector and miR-206 mimic (20 nM) 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). Scrambled control (Dharmacon) was used as the control for miR-206 mimic. 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.

miRNA transfection and gene expression

5×10^4 of HepG2 cells were seeded in a 24-well plate and allowed to adhere overnight. To determine the effects of miR-206 overexpression and knockdown on gene expression, HepG2 cells cultured in the DMEM with 10% FBS were transfected with MC-TTR-miR-206 (500 ng/well) or MC-TTR-miR-206-MM (500 ng/well) using Lipofectamine 3000. 24 hours after transfection, cells were washed using cold PBS and the total RNA were isolated for gene expression analysis.

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).

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 (11).

**Blood lipid analysis**
Blood was collected into tubes from cardiac puncture of C57Bl/6 mice. Serum 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 and analyzed by LICOR-Odyssey infra-red scanner. Primary antibodies of Ptpn1, py-PP2A and PP2A were purchased from Abcam; phosphorylated Insr, Insr, phosphorylated Irs1 and Irs1 were purchased from Santa Cruz.
Supplementary Table 1 Predicted targets of miR-206

| miRNA ID  | Gene Name | Species | Binding position | TargetScan Sites | picTar Sites |
|-----------|-----------|---------|------------------|------------------|--------------|
| mmu-miR-206 | HMGR   | mouse   | chr13:96649265-96649272[-] | 17[2]           | 17[2]        |
| hsa-miR-206 | CDK6   | human   | chr7:92243732-92243737[-] | 0[0]            | 294[1]       |
| hsa-miR-206 | CDK6   | human   | chr7:92241942-92241947[-] | 0[0]            | 14[2]        |
| hsa-miR-206 | CDK6   | human   | chr7:92240254-92240259[-] | 0[0]            | 29[2]        |
| hsa-miR-206 | CDK6   | human   | chr7:92239339-92239344[-] | 0[0]            | 320[3]       |
| hsa-miR-206 | CDK6   | human   | chr7:92237803-92237808[-] | 0[0]            | 1656[4]      |
| hsa-miR-206 | CDK6   | human   | chr7:92235488-92235495[-] | 333[10]         | 333[10]      |
| hsa-miR-206 | MET    | human   | chr7:116438225-116438230[+] | 0[0]            | 915[5]       |
| hsa-miR-206 | MET    | human   | chr7:116438034-116438039[+] | 0[0]            | 703[4]       |
| hsa-miR-206 | MET    | human   | chr7:116436992-116436998[+] | 433[3]          | 433[3]       |
| hsa-miR-206 | MET    | human   | chr7:116436677-116436683[+] | 437[1]          | 437[1]       |
| hsa-miR-206 | PTPN1  | human   | chr20:49200912-49200917[+] | 0[0]            | 215[10]      |
| hsa-miR-206 | PTPN1  | human   | chr20:49200425-49200430[+] | 0[0]            | 168[2]       |
| hsa-miR-206 | PTPN1  | human   | chr20:49200321-49200328[+] | 25[4]           | 25[4]        |
| hsa-miR-206 | NOTCH3 | human   | chr19:15271200-15271205[-] | 0[0]            | 12[1]        |
| hsa-miR-206 | CCND1  | human   | chr11:69467050-69467056[+] | 441[11]         | 441[11]      |

The numbers in the columns of Target Sites and picTar represent the Clip-Seq numbers (ClipSeq ReadNum) of miR-206 binding sites that are predicted through TargetScan or PicTar. The numbers of in the brackets represent the number in the ClipSeq peak cluster.
Supplementary Fig. 1 HFD treatment induces hepatosteatosis. (A) Oil-Red staining of livers from mice treated with either standard diet (SD) \((n=6)\) or high fat diet (HFD) \((n=6)\) for 8 weeks. (B) Hepatic lipid content in mice after 8 weeks of SD or HFD treatment. (C) Levels of serum triglycerides in mice treated with SD or HFD. (D) Fasting glucose levels of mice after 8 weeks of SD or HFD treatment. Data represent mean ± SEM. Mann-Whitney test was used for statistical analysis. \(P\) values are indicated.
Supplementary Fig. 2 Target Protector treatment impairs the capacity of miR-206 to inhibit expression of *PTPN1* in HepG2 Cells. (A) Expression levels of miR-206 and *PTPN1* in HepG2 cells transfected with MC-*TTR*-miR-206-MM (control), MC-*TTR*-miR-206, or a combination of MC-*TTR*-miR-206 and *PTPN1* TP. HepG2 cells were maintained in the DMEM medium containing 0.5 mM oleate. (B) Levels of miR-206 and *PTPN1* in HepG2 cells transfected with MC-*TTR*-miR-206-MM (control), MC-*TTR*-miR-206, or a combination of MC-*TTR*-miR-206 and *PTPN1* TP. HepG2 cells were cultured in the DEME containing gluconeogenic substrates including 20 mM sodium lactate and 2 mM sodium pyruvate in the presence of 1 nM insulin. MRNA levels were determined using qRT-PCR. Data represent mean ± SEM. Student T test was used to evaluate the statistical significance of miR-206 and *PTPN1*. *p < 0.05; **p < 0.01; and ***p < 0.001.
Supplementary Fig. 3  

*Ptpn1 mediates the role of miR-206 in inhibiting lipogenesis and facilitating insulin signaling pathway.*  

(A) miR-206 levels in livers of dietary obese mice treated with MC- TTR-miR-206 or MC- TTR-miR-206-MM (control).  

(B) Expression levels of miR-206 and Ptpn1 in livers of HFD-treated mice after treatment of MC- TTR-miR-206 or a combination of MC- TTR-miR-206 and MC- TTR-Ptpn1. The control group (n=9) received MC- TTR-miR-206-MM.  

*Ptpn1* and miR-206 expression was determined using qRT-PCR. Data represent mean ± SEM. Student T test was used to evaluate the statistical significance of miR-206 and *Ptpn1*. **p < 0.01 and ***p < 0.001.
Supplementary Fig. 4 MC-\textit{TTR}\textndash miR-206 treatment has no effect on mRNA levels of the genes controlling fatty acid oxidation in livers of dietary obese. qRT-PCR was used to determine mRNA levels of these genes. Data represent mean ± SEM. Student T test was used for statistical analysis.
Supplementary Fig. 5 mRNA levels of genes controlling cholesterol transfer and synthesis in livers of miR-206-treated mice and macrophages. qRT-PCR was used to determine mRNA levels of these genes. Data represent mean ± SEM. (A) mRNA levels of Lxrα, Abca1, Abcg1, ApoE, Hmgcr and Srebp1c in livers of mice treated with MC-TTR-miR-206-MM or MC-TTR-miR-206. (B) Lxrα, Abca1, Abcg1, and ApoE in macrophage cells treated with MC-TTR-miR-206-MM (control) or MC-TTR-miR-206. Data represent mean ± SEM. Student T test was used for statistical analysis. *p < 0.05; **p < 0.01; and ***p < 0.001.
Supplementary Fig. 6 miR-206 levels in the livers of mice. Eight-week-old wild-type male C57Bl/6 mice (Jackson Laboratory, n=6) received one injection of MC-TTR-miR-206 in a volume of 100 µl saline (2 µg/g body weight). After 2 weeks, mice were sacrificed for liver collection and miR-206 expression analysis. Control mice (n=6) were injected with MC-TTR-miR-206-MM. Data represent mean ± SEM. Mann-Whitney test was used to evaluate the statistical significance. *p < 0.05
Supplementary Fig. 7 Both mini-circle vector and MC-TTR-miR-206 have negligible effects on ALT and AST. (A) ALT levels, and (B) AST levels. Eight-week-old wild-type male C57Bl/6 mice (Jackson Laboratory, n=6) were maintained on a high fat diet (Open Source D12492: 60% Kcal fat) for 8 weeks. After 8 weeks of HFD administration, mice were injected with eight 0.9% saline (n=6), MC-TTR empty vector or MC-TTR-miR-206 weekly for another 8 weeks. At 24 weeks of age, mice were sacrificed for serum chemistry analysis. Data represent mean ± SEM. Mann-Whitney test was used for statistical analysis. P values are indicated. NS: no significance.
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