miR-22 inhibition reduces hepatic steatosis via FGF21 and FGFR1 induction

Ying Hu, Hui-Xin Liu, Prasant Kuma Jena, Lili Sheng, Mohamed R. Ali, Yu-Jui Yvonne Wan

Table of contents

Supplementary materials and methods

RNA isolation and gene expression quantification: Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA was generated using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA, USA) as described previously (2, 3). qRT-PCR was performed at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute on ABI 7900HT Fast Real-time PCR system using Power SYBR® Green PCR Master Mix (Applied Biosystems). For each sample, GAPDH expression was analyzed to normalize target gene expression. Primers for qRT-PCR were designed with Primer3 Input software (version 0.4.0).

To determine levels of mature miRNA expression, total RNA was used for miRNA-specific cDNA synthesis with the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). qRT-PCR was performed on an ABI 7900HT Fast Real-time PCR system using Taqman MicroRNA Assays Kit (Thermo Fisher Scientific) for mature *miR-22* or U6. The small RNA U6 was used to normalize *miR-22* expression.

Reporter vector construction and luciferase reporter assay: The 3' untranslated region (3'-UTR) of the *FGFR1* gene containing the putative binding site for *miR-22* (500 bp) and the entire 3'-UTR of the *FGF21* gene (105 bp) was amplified from human genomic DNA (Promega, Madison, WI, USA) and cloned into the psiCHECK2 vector (Promega) using NotI and XhoI. For Huh7 cell infection, adenovirus-GFP (negative control), adenoviral-*miR-22*-GFP, or adenoviral-*miR-22* inhibitor-GFP (Applied Biological Materials Inc.) were used. Forty-eight h post-infection, the infected Huh7 cells were transfected with psiCHECK2-*FGR1*-3'UTR or psiCHECK2-*FGF21* 3'UTR using Lipofectamine 2000 (Thermo Fisher Scientific). Twenty-four hours later, cells were harvested for firefly and renilla luciferase assay using the Dual-luciferase Reporter system

(Promega) as described previously (2, 3). Renilla luciferase activity was standardized to firefly luciferase activity.

Hepatic lipid analysis: Hepatic triglyceride and cholesterol were measured as previously described (4). Briefly, 100 mg frozen tissues were homogenized in a 1-ml solution containing 5% NP-40 (MilliporeSigma, Burlington, MA, USA) in water. Samples were heated to 80°C for 5 min and cooled to room temperature followed by centrifugation for 5 min at 13,000 X g to remove insoluble material. Hepatic triglycerides and cholesterol were quantified from the supernatants using a colorimetric assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's protocols.

Serum FGF21 and glucagon-like peptide-1 (GLP-1) secretion assay: Serum FGF21 levels were quantified by ELISA (Boster Biological Technology, Pleasanton, CA, USA). For GLP-1 secretion assays, mice were fasting for 12 h followed by oral administration of dipeptidyl peptidase-4 inhibitor, sitagliptin (3 mg/g body weight; TSZ Chem, Framingham, MA, USA) and a liquid diet (Ensure Plus, 10ml/g body weight; Ross Laboratories, Columbus, OH, USA). The liquid diet consisted of 15% protein, 57% carbohydrate, and 28% fat to stimulate GLP-1 secretion, as previously described (5). Blood samples were collected immediately (time 0) as well as 15, and 30 min after Ensure Plus feeding. Serum GLP-1 was quantified by an ELISA kit (RayBiotech Life, Norcross, GA, USA).

Serum biochemistry analysis: Blood samples were collected at the endpoint of the experiments and serum was separated within 2 h of collection after centrifugation at 3,000 X g for 10 min. Serum lipopolysaccharide (LPS) (Thermo Fisher Scientific), alanine transaminase

(ALT) (Pointe Scientific), and alkaline phosphatase (ALP) (Pointe Scientific) levels were quantified according to the manufacturer's instructions as described previously (4).

Insulin tolerance test: Insulin tolerance was measured as previously described (5). After 6 h food deprivation, tail vein blood was used to establish fasting blood glucose levels. For insulin tolerance testing, insulin (1 U/kg body weight, i.p.; MilliporeSigma) was given followed by measuring blood glucose level at various times with the OneTouch Ultra 2 (Johnson & Johnson Co., New Brunswick, NJ, USA). The area under the curve (AUC) of the blood glucose levels over time was calculated.

Chromatin immunoprecipitation-qPCR: Chromatin immunoprecipitation-qPCR was performed based on a previous publication (6). Briefly, chromatin lysate was precleared before incubation with anti-PGC1α (Novus Biologicals) and anti-PPARα (Santa Cruz Biotechnology). Rabbit IgG and RNA Polymerase II antibody (MilliporeSigma) were used as negative and positive controls, respectively. Samples were incubated with Dynase beads at 4ºC overnight followed by de-crosslinking and purification. DNA fragments generated served as templates for qPCR using Power SYBR® Green PCR Master Mix (Applied Biosystems).

Western blot and antibodies: Western blots were performed as described previously (2, 3). Proteins were extracted from the cell and livers using lysis buffer with cock-tail proteases inhibitors and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentration was measured using Pierce BCA protein assay kit and 20~40 μg of total lysate was loaded and immunoblotted. Antibodies used were anti-FGFR1, FASN, phosphor (P)-AMPK, total (T)-AMPK, phosphor (P)-ERK1/2, and total (T)-ERK1/2 (Cell Signaling Technology, Beverly, MA, USA), FGF21 (Abcam, Cambridge, MA, USA), β-ACTIN (MilliporeSigma), CD36 (Bioss Antibodies Inc.,

Woburn, MA, USA), CYCLIN A2, PGC1α (Novus Biologicals, LLC, Littleton, CO, USA), and PPARα (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunohistochemistry: To monitor the proliferative effect of *miR-22* inhibitor treatment, immunostaining was performed with anti-Ki-67 antibody (NeoMarkers, Fremont, CA, USA) in the livers obtained from healthy mice and WD-fed mice based on a previous publication (7). The number of proliferating hepatocytes was determined by counting positively stained cells in at least 5 random microscopic fields (40X) per mouse liver. Three-month-old male and female WT mice received adenovirus (negative control) or *miR-22* inhibitor ($1x10^9$ PFU, tail vein injection, once a week) for 4 months. Untreated age- and sex-matched WT mice were used as baseline controls. In addition, Ki-67 immunostaining was also performed in WD-fed mice treated with the *miR-22* inhibitor, OCA, and the combination of both as well as adenovirus, which served as a negative control. Untreated age- and sex-matched mice were included as baseline controls.

References

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Supplemental Table S1. Human liver specimens used in this study. The human fatty livers and normal livers with fat content of <5% were obtained from the Gastrointestinal Biorepository at UC Davis. Steatosis was graded by a pathologist from 0 to 3 based on fat content: grade 0 (normal) ≤ 5%, grade 1 (mild) = 5%~33%, grade 2 (moderate) = 34%~66%, and grade 3 (severe) ≥ 67% of hepatocytes having lipid.

Subject No.	Steatosis (%)	Steatosis grade	Race	Sex	BMI	Alcohol (ever)	Alcohol (ended)	Alcohol consumption Frequency
1	2%	0	Caucasian	Female	37.9	Current	N/A	Weekly
2	<5%	0	Caucasian	Female	60.1	Former	2015	Yearly
3	<5%	0	Caucasian	Female	40.4	Former	2010	Weekly
4	2%	0	African-American	Female	41.3	Former	Unreported	Monthly
5	5%	0	Caucasian	Female	39.5	Current	N/A	Weekly
6	2%	0	Caucasian	Female	41.9	No	N/A	N/A
7	<2%	0	African-American	Female	62.3	Current	N/A	Monthly
8	5%	0	Caucasian	Female	39.5	Current	N/A	Weekly
9	10%	1	Caucasian	Female	50.4	Current	N/A	Yearly
10	20%	1	Caucasian	Female	43.9	Current	N/A	Yearly
11	10%	1	Caucasian	Female	49.4	Former	2010	Yearly
12	15%	1	Caucasian	Female	42.9	Current	N/A	Monthly
13	10%	1	> one race	Female	48.2	Current	N/A	Monthly
14	25%	1	Caucasian	Female	48.6	Former	2012	Daily
15	25%	1	> one race	Female	54.5	Current	N/A	Monthly
16	20%	1	African-American	Female	49.9	Former	1987	Yearly

17	40%	2	Caucasian	Female	35.2	Current	N/A	Weekly
18	70%	3	African-American	Female	49.9	Former	2015	Weekly
19	50-60%	2	Caucasian	Female	40.6	Current	N/A	Weekly
20	50%	2	Caucasian	Female	51.4	Current	N/A	Yearly
21	30-40%	2	Caucasian	Female	30.3	Former	Unreported	Weekly
22	40%	2	Caucasian	Male	45.0	No	N/A	N/A
23	35%	2	Caucasian	Male	39	Former	2010	Weekly
24	50-60%	2	Caucasian	Female	35.8	Former	2015	Monthly
25	40-50%	2	Caucasian	Female	47.8	No	N/A	N/A

Fig. S1. The relative expression of genes in enriched hepatocytes (Hepa) and liver NPCs illustrates the efficacy of the methods. The expression levels of cell type specific markers in hepatocytes (Hepa) and NPC of CD- or WD-fed male mice. Data = mean ± SD (n=4). * p<0.05, ** p<0.01, *** p<0.001. CD, control diet; NPCs, non-parenchymal cells; WD, Western diet.

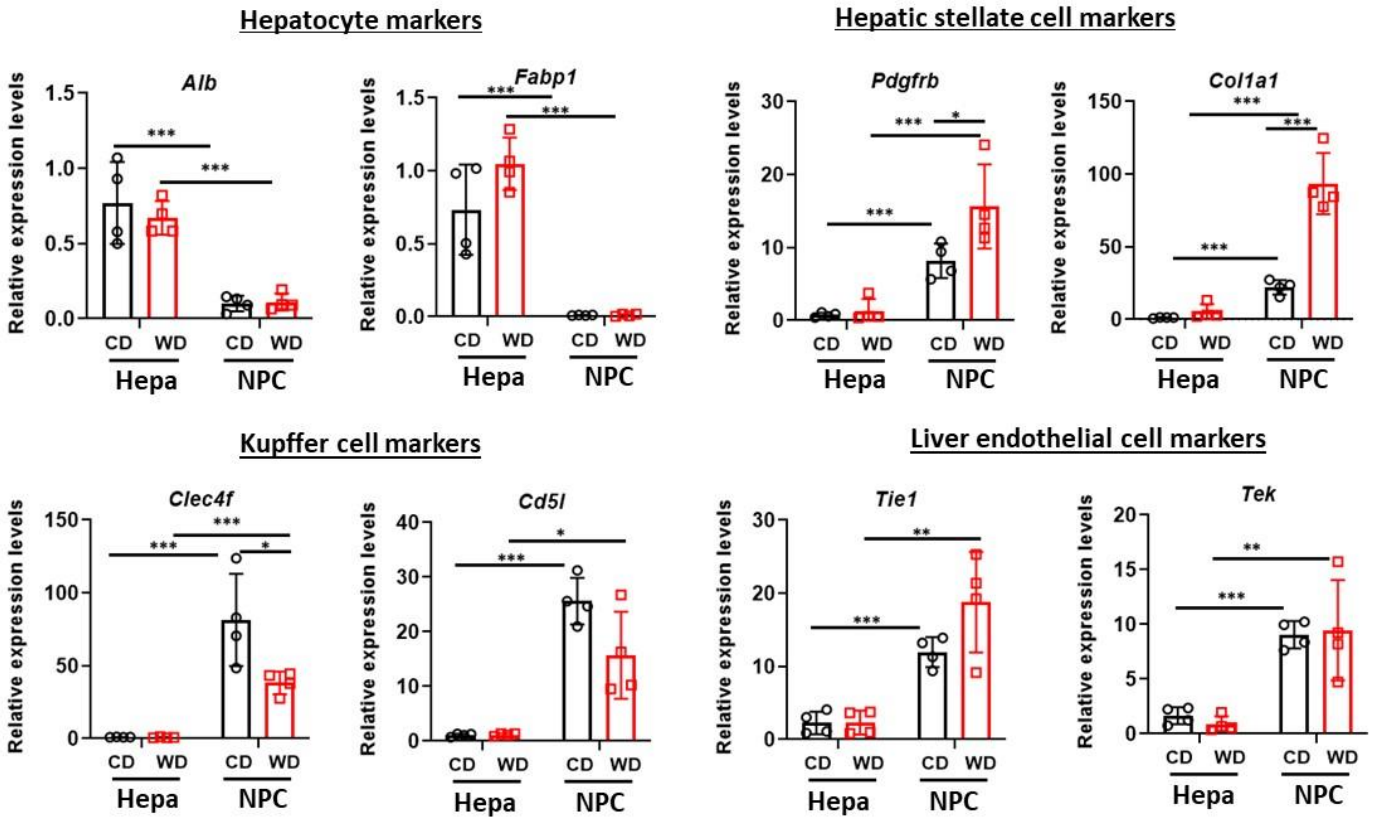

Fig. S2. The *miR-22* inhibitor does not have a proliferative effect or apparent toxicity in healthy mice. Three-month-old male and female C57BL/6 healthy mice received adenovirus control or *miR-22* inhibitor ($1x10^9$ PFU, tail vein injection, once/week) for 4 months. Age- and sex-matched mice without any treatment were used as baseline controls. All the mice were euthanized 1 day after the last viral injection. (A) Body weight gain, liver-to-body weight ratio, blood glucose level after 6 h fasting; serum ALT, ALP, and endotoxin (LPS) levels; (B) representative liver sections of Ki-67 staining; Ki-67-positive cells were counted in 5 random fields (40X) per liver section (3 mice per group). Scale bar indicates 100 μm. Data are expressed as mean ± SD. One-way ANOVA with Tukey's *t*-test. ** $p<0.01$. ALP, alkaline phosphatase; ALT, alanine aminotransferase; LPS, lipopolysaccharide; PFUs, plaque-forming units.