Alvarez-Sola et al.

Supplementary Materials and Methods

Cell culture and treatments

The human well-differentiated hepatoblastoma cell line HepG2 and the human hepatocarcinoma cell line Hep3B (from ATCC) were cultured as described.[1] The immortalized human biliary epithelial cell line H69 (kindly provided by Dr. Gregory Gores, Mayo Clinic, Rochester, MN, USA) was grown as reported.[2] Where indicated cells were treated with palmitic acid (PA) (Sigma, St. Louis, MO, USA) dissolved in isopropanol and added to serum-free culture media containing 1% fatty acid free BSA to the desired final concentration as reported,[3] controls received same volume of isopropanol (always <1% in the medium). For DNMT1 knockdown HepG2 cells were transfected with DNMT1 specific siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or with control siRNAs (siGL) for 48 h as previously described.[1] 5-aza-deoxycytidine (5AZAdC) was from Sigma. For the study of *PPARγ2* gene expression regulation, where indicated HepG2 cells were treated with a lipogenic cocktail (DMI): 0.5 mM 3-isobutyl-1-methyl-xanthine IBMX, 10 μg/ml insulin and 1 μM dexamethasone in serum free medium, with or without FGF19 (50 ng/ml), for 48 h as described. [4,5] Subsequently cells were further incubated in the presence of insulin (10μg/ml) with or without FGF19 (50 ng/ml) for another 60 h, and then lysed for mRNA extraction and analyses. For the analysis of p70S6 kinase (p70S6K) phosphorylation, Hep3B cells were serum-starved for 6h in DMEM medium supplemented with 0.2% bovine serum albumin. Then, where indicated cells were pretreated with the FGFR4 inhibitor BLU9931 (Cayman Chemical, MI, USA) for 1h before being stimulated with ApoA-I, FGF19 or Fibapo for 30 min.

Apoptosis Assay

Nucleosomal fragmentation characteristic of apoptosis was estimated by the determination of soluble histone-DNA complexes using the Cell Death Detection ELISA Assay (Roche Applied Sciences, Germany) as previously described.[6]

Western blot analyses

Cells and liver tissues were lysed in RIPA buffer and homogenates were subjected to western blot analysis as reported.[1,7] Antibodies used were: anti-GRP78 (Bip), anti-CHOP, anti-cleaved caspase-3 (Asp 175), anti-p-p70S6K (Thr 389), anti-p70S6K, anti-p-S6 (Ser 235/236), anti-S6, anti-β-ACTIN and anti-α-Tubulin from Cell Signaling Technology (Beverly, MA, USA); anti-PPARγ2 and anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-FGF19 from R&D Systems (Minneapolis, MN, USA); anti-CD36 from GeneTex (Irvine, CA, USA).

Sandwich ELISA for the determination of FGF19 and Fibapo protein levels

Serum samples, tissue homogenates (10 mg in 500 μl of RIPA buffer) and cell culture conditioned media were incubated for 24h at 4 °C with anti-FGF19 antibody (8μg/ml) as capture antibody and for 4h RT with biotinylated anti-FGF19 antibody (100 ng/ml) (Refs. AF969 and BAF969, both from R&D Systems, Minneapolis, MN, USA). A standard curve using recombinant human FGF19 was used to calculate protein concentrations after subtraction of background signal calculated from non-FGF19/Fibapo treated samples (in serum and tissue samples). For the calculation of FGF19 and Fibapo serum t1/2, protein serum concentrations over time were fitted to the equation for one-compartment model after an *i.v.* bolus administration and the estimated elimination constants were compared with the Extra sum-of-squares F test. GraphPad Prism

software (GraphPad Software Inc., San Diego, USA) version 7.00 was employed for statistical analysis.

Cloning of FGF19 and Fibapo and construction of AAVs

FGF19 and Fibapo cDNAs, synthetized by GenScript (Piscataway, NJ, USA), were subcloned into the pcDNA3.1 mammalian expression vector for hydrodynamic injection experiments. FGF19, ApoA-I and Fibapo cDNAs were also cloned in adenoassociated viral vectors (AAV8) flanked by AAV2 WT inverted terminal repeats, and under the regulation of a chimeric liver-specific promoter composed of the human α1-antitrypsin promoter (AAT) with regulatory sequences from the albumin enhancer (Eal) as described before.[1] All rAAV8 viruses were produced by polyethyleneimine-mediated (PEI-mediated) cotransfection with pDP8.ape plasmid (PlasmidFactory GmbH & Co.) in HEK-293 cells.[8] Cells were harvested 72 h after transfections and virus was released from the cells by three rounds of freeze-thawing. Crude lysate from all batches was then treated with RNase and DNase for 1 h at 37ºC and kept at - 80º C until purification, which was performed by iodixanol gradients as described.[9] The purified batches were concentrated and diafiltrated by cross-flow filtration with a molecular mass cutoff of 400 kDa. Batches were then concentrated further by passage through Centricon tubes (YM-100; Millipore) to a final concentration of 1 x 10^{12} vg/ml, as determined by qPCR. Finally, viral batches were filtered (pore size 0.22 mm) and stored at -80 ºC. Mice were intravenously infected with 1 x 10^9 vg in the case of *Fgf15*$^{-/-}$ mice (≈25 g body weight) or 5 x 10^9 vg for db/db mice (≈45 g body weight).

Production of recombinant FGF19 and Fibapo proteins

FGF19, Fibapo and ApoA-I were synthesized upon request by GenScript (Piscataway, NJ, USA). For Fibapo synthesis FGF19 sequence was fused to the N-terminus of human apolipoprotein A-I

with a short linker (GAP). To facilitate purification of the proteins a His-tag was added to the N-terminus end of both proteins. The amino acid sequences of FGF19 and Fibapo are provided in supplementary figure S5. The recombinant proteins were expressed in *E. coli* and purified from inclusion bodies by Ni^{2+}-affinity chromatography by the manufacturer. Endotoxin levels were below 1EU/μg.

Isolation of HDL

HDL isolation from serum was performed by differential ultracentrifugation in sodium bromide gradient as described.[10]

RNA isolation and qPCR

Total RNA from liver and ileal tissues, and cell lines, was extracted using the automated Maxwell system from Promega (Madison, WI, USA). Reverse transcription was performed as described.[1] Real-time PCRs were performed with iQ SYBR Green supermix (BioRad) in a CFX96 system from BioRad as previously described.[1] Primers are described in Supplementary Table 1.

Hepatic triglyceride content determination

Intrahepatic TG concentration was measured by saponification in ethanolic KOH as described [11,12] by determining triolein equivalents using a commercially available kit (BQ029A-CR, BQ Kits, San Diego, CA, USA).

Total bile acid determination

Liver tissues were homogenated in water (5 mg/500 μl), sonicated and centrifuged at 10,000g for 10 min. BAs concentrations were determined in supernatants by an enzymatic/colorimetric method using a commercially available kit (Randox Laboratories, Crumlin, UK).

Histological determinations

Liver tissue samples were formalin-fixed and paraffin-embedded for H&E staining and the degree of tissue necrosis was evaluated and scored as previously reported.[7] For lipids staining in frozen tissue sections Oil Red O (Sigma) was used as previously published.[13]

Ki67 immunohistochemistry

Positive Ki67 hepatocyte number was determined by immunohistochemistry as previously described [7] in 5 liver fields per mice (5x amplification) using ImageJ software (NIH, Bethesda, Maryland, USA).

Serum biochemistry and BA determinations

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin and BA were measured as previously reported.[7,14]

Patients description and analyses

The control and obese patients analyzed in this study have been described previously.[15] Briefly, liver tissue samples from 69 obese subjects (16 males and 53 females) were obtained from patients attending the Departments of Endocrinology & Nutrition and Surgery at the Clínica Universidad de Navarra, Pamplona, Spain. Patients underwent a clinical assessment including medical history, physical examination, body composition analysis and co-morbidity evaluation by

a multidisciplinary consultation team. BMI was calculated as weight in kilograms divided by the square of height in meters and body fat (BF) was estimated by air-displacement-plethysmography (Bod-Pod®, Life Measurements, Concord, CA).[16] The waist-to-hip ratio (WHR) was measured as the quotient between the circumference of the waist (at the midway level between the margin of the lowest rib and the iliac crest) and the hip (at the widest trochanters). Obese patients were further subclassified according to the established diagnostic thresholds for diabetes [normoglycemia (NG): fasting plasma glucose (FPG) concentration < 100 mg/dL and PG < 140 mg/dL 2-h after an oral glucose tolerance test (OGTT) and T2D: FPG > 126 mg/dL or PG ≥ 200 mg/dL 2-h after OGTT)].[17] T2D subjects were not on insulin therapy or on medication likely to influence endogenous insulin levels. The intraoperative liver biopsy was obtained from obese patients during bariatric surgery for morbid obesity treatment to establish a histological diagnosis of the hepatic state. The diagnosis of non-alcoholic fatty liver disease was established following a histopathologic evaluation.[18] Liver tissue samples were also obtained from lean healthy controls (BMI $\leq$ 25 kg m^{-2}) undergoing liver biopsies (when hepatic tumor was suspected and results were negative, or during hiatal hernia repair; a total of 18 patients). Tissue samples were immediately frozen in liquid nitrogen and stored at -80 ºC for subsequent analyses. Serum was obtained from blood drawn from patients after a 12 h overnight fast. The study was approved by the University of Navarra Ethical Committee and the written informed consent of participants was obtained. The biochemical and hormonal characteristics of the obese subjects included in the study are shown in Supplementary Table 2.

Serum FGF19 and hepatic *PPARγ2* mRNA levels were determined as reported [15] using the primers indicated in Supplementary Table 1. Since no significant differences were found in the circulating FGF19 levels, nor in liver *PPARγ2* mRNA levels, between the two groups of obese patients (normoglycemic and T2D+impaired glucose tolerance), these data were combined in our analysis.

Statistical analysis

Data are means ± SEM. Data were compared using the Student *t* test. A *p* value of <0.05 was considered significant. For the analysis of mouse survival in the 85% PH study *p*-value was calculated using the Mantel-Cox (log-rank) test. Data analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, USA) version 7.0 was employed for statistical analyses.

References

1 Elizalde M, Urtasun R, Azkona M, *et al.* Splicing regulator SLU7 is essential for maintaining liver homeostasis. *J Clin Invest* Published Online First: 27 May 2014. doi:10.1172/JCI74382

2 Grubman SA, Perrone RD, Lee DW, *et al.* Regulation of intracellular pH by immortalized human intrahepatic biliary epithelial cell lines. *Am J Physiol* 1994;**266**:G1060–70.

3 Natarajan SK, Ingham SA, Mohr AM, *et al.* Saturated free fatty acids induce cholangiocyte lipoapoptosis. *Hepatology* 2014;**60**:1942–56. doi:10.1002/hep.27175

4 Schadinger SE, Bucher NLR, Schreiber BM, *et al.* PPARgamma2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes. *Am J Physiol Endocrinol Metab* 2005;**288**:E1195–205. doi:10.1152/ajpendo.00513.2004

5 Lai P-H, Wang W-L, Ko C-Y, *et al.* HDAC1/HDAC3 modulates PPARG2 transcription through the sumoylated CEBPD in hepatic lipogenesis. *Biochim Biophys Acta* 2008;**1783**:1803–14. doi:10.1016/j.bbamcr.2008.06.008

6 BERASAIN C, García-Trevijano ER, Castillo J, *et al.* Novel role for amphiregulin in protection from liver injury. *J Biol Chem* 2005;**280**:19012–20. doi:10.1074/jbc.M413344200

7 Uriarte I, Fernández-Barrena MG, Monte MJ, *et al.* Identification of fibroblast growth factor 15 as a novel mediator of liver regeneration and its application in the prevention of post-resection liver failure in mice. *Gut* 2013;**62**:899–910. doi:10.1136/gutjnl-2012-302945

8 Gil-Fariña I, Di Scala M, Vanrell L, *et al.* IL12-mediated liver inflammation reduces the formation of AAV transcriptionally active forms but has no effect over preexisting AAV transgene expression. *PLoS ONE* 2013;**8**:e67748. doi:10.1371/journal.pone.0067748

9 Zolotukhin S, Byrne BJ, Mason E, *et al.* Recombinant adeno-associated virus purification

using novel methods improves infectious titer and yield. *Gene Ther* 1999;**6**:973–85. doi:10.1038/sj.gt.3300938

10 Fioravanti J, González I, Medina-Echeverz J, *et al.* Anchoring interferon alpha to apolipoprotein A-I reduces hematological toxicity while enhancing immunostimulatory properties. *Hepatology* 2011;**53**:1864–73. doi:10.1002/hep.24306

11 Salmon DM, Flatt JP. Effect of dietary fat content on the incidence of obesity among ad libitum fed mice. *Int J Obes* 1985;**9**:443–9.

12 Norris AW, Chen L, Fisher SJ, *et al.* Muscle-specific PPARgamma-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidinediones. *J Clin Invest* 2003;**112**:608–18. doi:10.1172/JCI17305

13 Mehlem A, Hagberg CE, Muhl L, *et al.* Imaging of neutral lipids by oil red O for analyzing the metabolic status in health and disease. *Nat Protoc* 2013;**8**:1149–54. doi:10.1038/nprot.2013.055

14 Garcia-Irigoyen O, Carotti S, Latasa MU, *et al.* Matrix metalloproteinase-10 expression is induced during hepatic injury and plays a fundamental role in liver tissue repair. *Liver Int* Published Online First: 4 October 2013. doi:10.1111/liv.12337

15 Gallego-Escuredo JM, Gómez-Ambrosi J, Catalan V, *et al.* Opposite alterations in FGF21 and FGF19 levels and disturbed expression of the receptor machinery for endocrine FGFs in obese patients. *Int J Obes (Lond)* 2015;**39**:121–9. doi:10.1038/ijo.2014.76

16 Ginde SR, Geliebter A, Rubiano F, *et al.* Air displacement plethysmography: validation in overweight and obese subjects. *Obes Res* 2005;**13**:1232–7. doi:10.1038/oby.2005.146

17 Genuth S, Alberti KGMM, Bennett P, *et al.* Follow-up report on the diagnosis of diabetes mellitus. 2003. 3160–7.

18 Brunt EM. Nonalcoholic steatohepatitis. *Semin Liver Dis* 2004;**24**:3–20. doi:10.1055/s-2004-823098