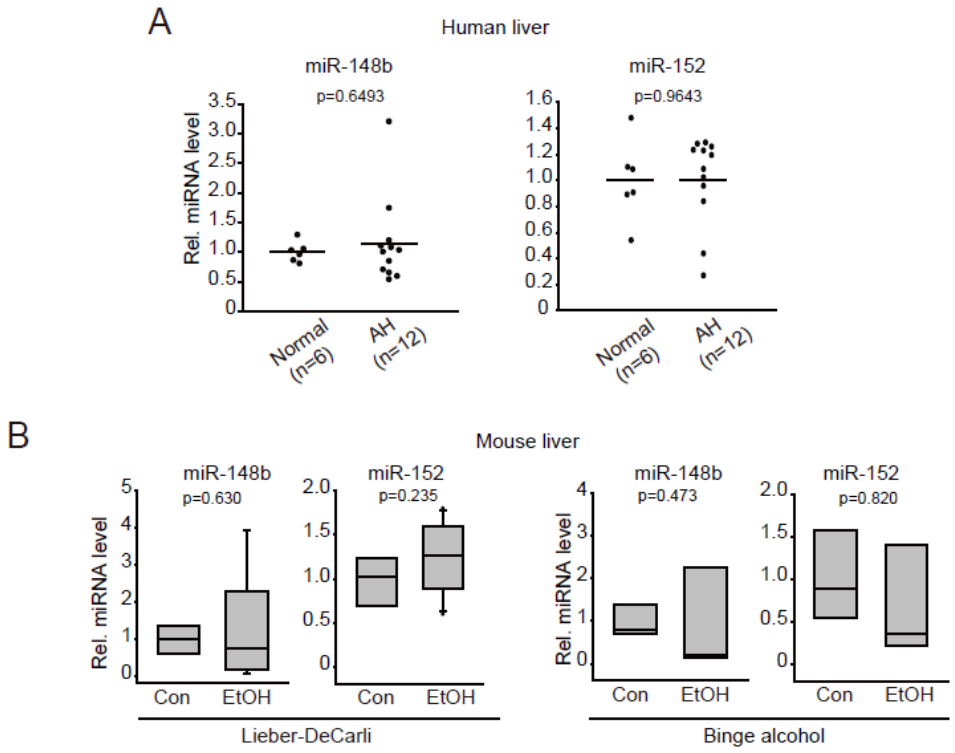
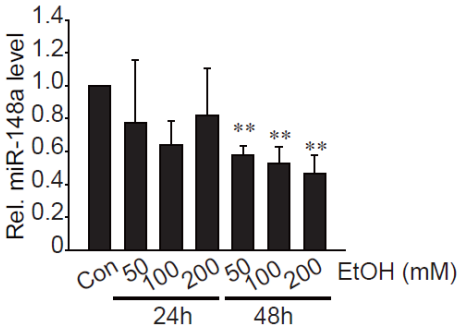


Supplementary Figure 1

Supplementary Figure 1. miR-148b and miR-152 levels in human or mouse liver samples

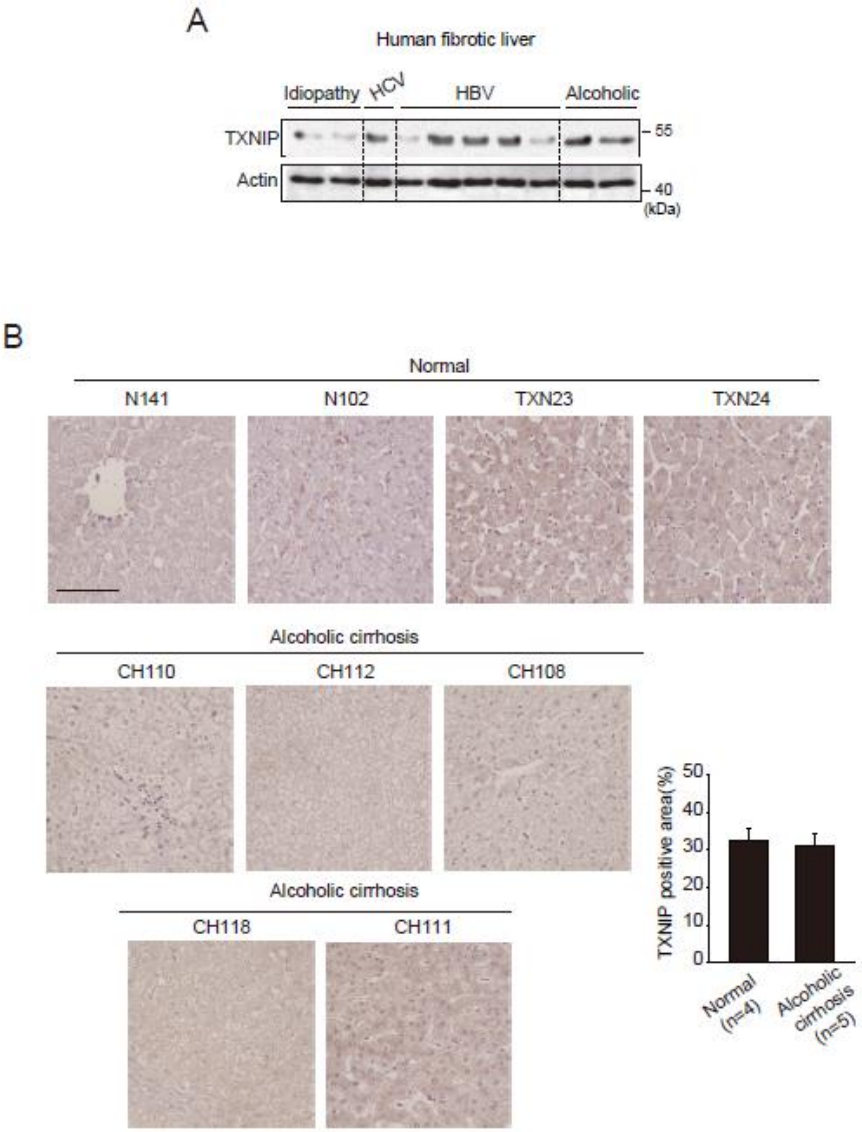
(A) qRT-PCR assays for miR-148b and miR-152 in the liver of normal subjects or AH patients (n=6 or 12 each) as used in Figure 1B. Each point represents one sample, and the horizontal line indicates the mean value.

(B) qRT-PCR assays for miR-148b and miR-152 in mouse liver samples as used in Figure 1C. Mice were fed Lieber-DeCarli alcohol liquid diet for 6 weeks (n=6 or 10 each), or binge alcohol gavage for 7 days (n=7 each). Data were shown as box and whisker plot. Box, interquartile range (IQR); whiskers, 5–95 percentiles; and horizontal line within box, median. Con, control diet; and EtOH, alcohol-containing diet



Supplementary Figure 2

Supplementary Figure 2. qRT-PCR assays for miR-148a in AML-12 cells treated with ethanol at the indicated time and concentration. Data represents the mean ± SEM (n=3). Significantly different as compared to control (Con), **p<0.01

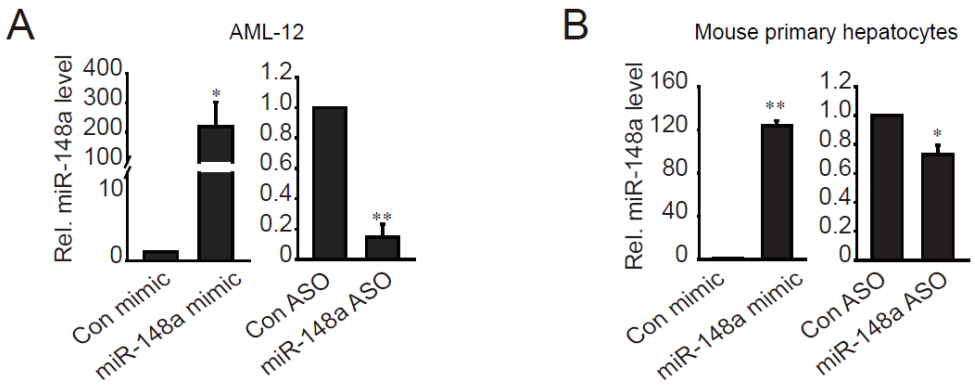


Supplementary Figure 3

Supplementary Figure 3. TXNIP levels in liver specimens from patients with fibrosis or cirrhosis

(A) Immunoblotting for TXNIP in liver specimens from fibrosis patients with different etiologies.

(B) Immunohistochemistry for TXNIP in liver specimens from patients with alcoholic cirrhosis. Data represents the mean ± SEM (n=4 or 5 each). Scale bar, 100 μm



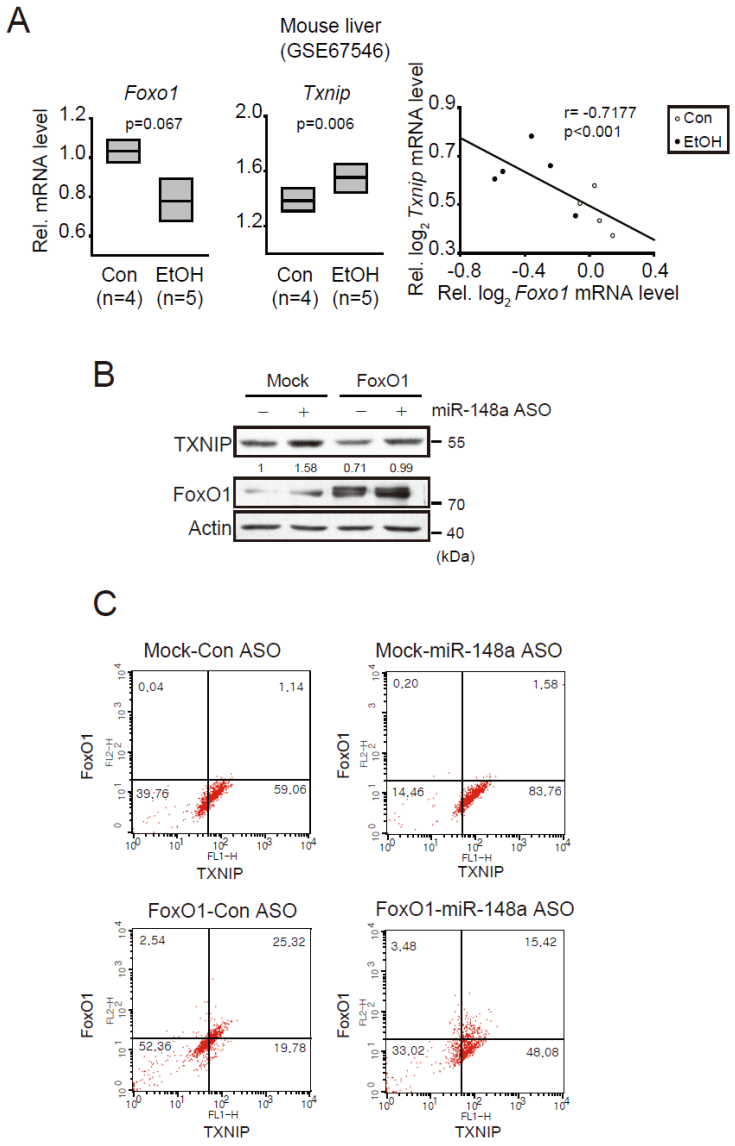
Supplementary Figure 4

Supplementary Figure 4. qRT-PCR assays for miR-148a

(A) AML-12 cells were transfected with the indicated mimic for 48 h, or with ASO for 72 h.

(B) Primary hepatocytes isolated from mice that had been treated with binge alcohol for 7 days were transfected as above.

For A and B, data represents the mean ± SEM (n=3 or 4 each). Significantly different as compared to respective control, *$p<0.05$, **$p<0.01$



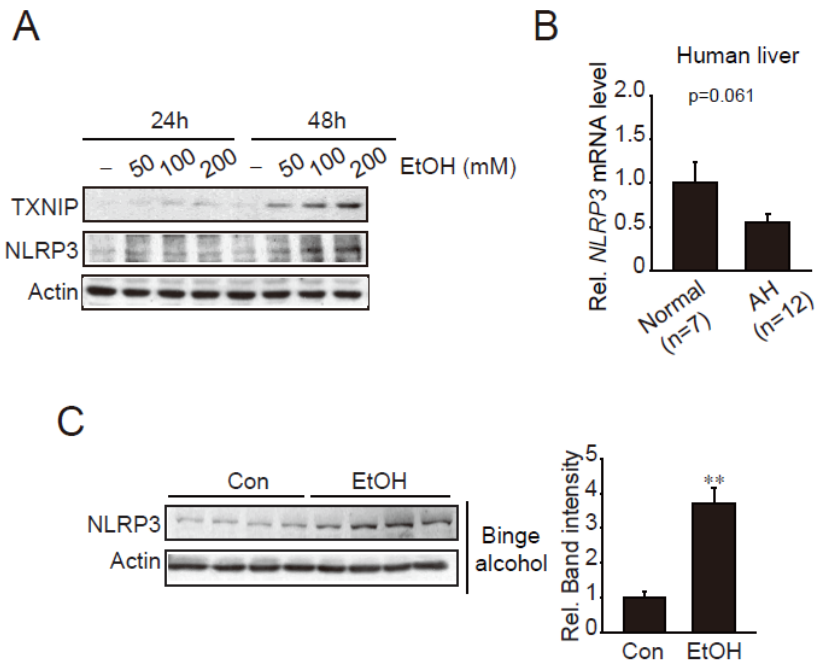
Supplementary Figure 5

Supplementary Figure 5. The role of FoxO1 in the regulation of TXNIP by miR-148a

(A) FoxO1 and TXNIP mRNA levels in the liver. cDNA array dataset (GSE67546) from control mice (Con, n=4) and those fed on Lieber-DeCarli ethanol diet (EtOH, n=5) was used to assess transcript levels and the correlation. Data were shown as box and whisker plot. Box, interquartile range (IQR); whiskers, 5−95 percentiles; and horizontal line within box, median

(B) Immunoblotting for TXNIP in the lysates of AML-12 cells exposed to 100 mM ethanol for 48 h after transfection with the plasmid encoding FoxO1 (or Mock) in combination with miR-148a ASO (or Con ASO) for 24 h. Values indicate intensities relative to Mock control.

(C) Flow cytometry for TXNIP and FoxO1. AML-12 cells were treated as above for flow cytometric assays.



Supplementary Figure 6

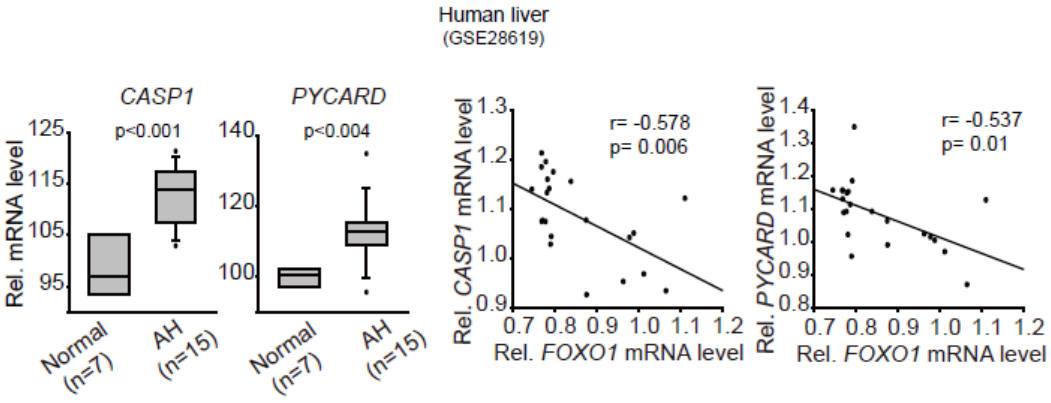
Supplementary Figure 6. TXNIP and NLRP3 protein or transcript levels

(A) Immunoblottings for TXNIP and NLRP3 in AML-12 cells treated with ethanol at the indicated time and concentration.

(B) NLRP3 mRNA levels in normal subjects or AH patients (n=7 or 12 each).

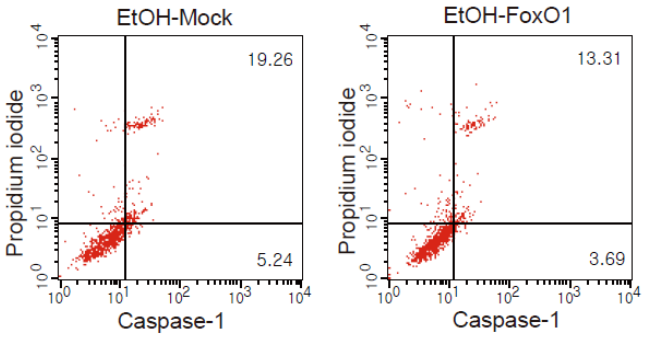
(C) Immunoblotting for NLRP3 in the liver from mice subjected to binge alcohol treatment (n=4 each).

For B and C, data represents the mean ± SEM. Significantly different as compared to control, **p<0.01



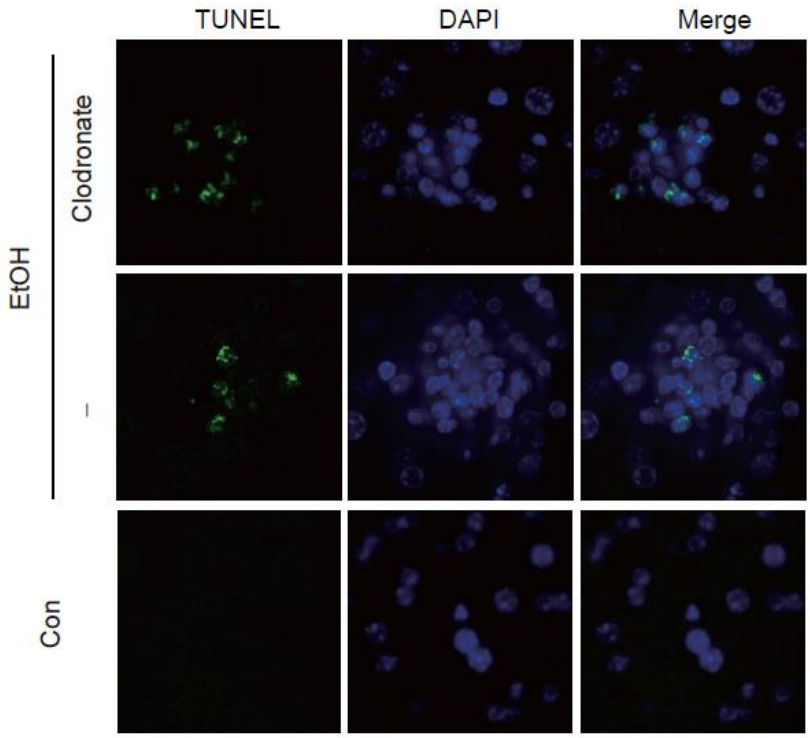
Supplementary Figure 7

Supplementary Figure 7. Caspase-1 (*CASP1*) and ASC (*PYCARD*) transcript levels in healthy individuals (Normal) or AH patients (AH), and their correlation analyses with FoxO1. The mRNA levels were obtained from cDNA array dataset (GSE28619) available in public domain. Data were shown as box and whisker plot. Box, interquartile range (IQR); whiskers, 5−95 percentiles; and horizontal line within box, median



Supplementary Figure 8

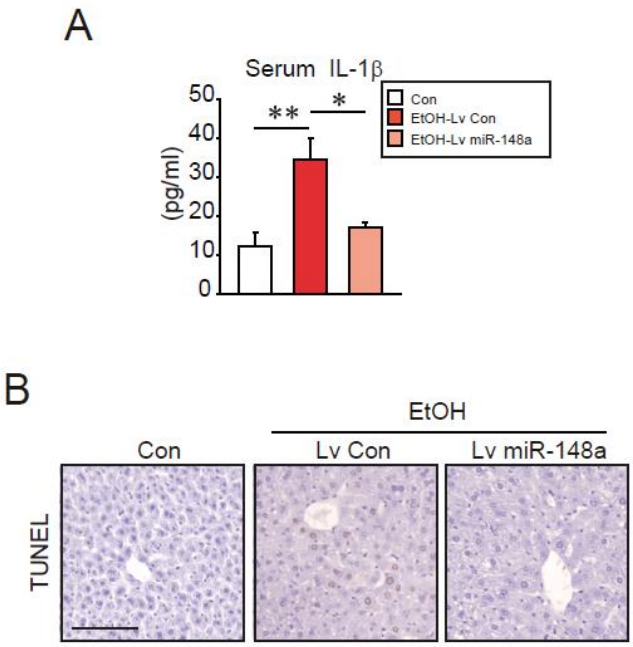
Supplementary Figure 8. Inhibition of ethanol-induced hepatocyte pyroptosis by FoxO1 overexpression AML-12 cells were treated with ethanol for 48 h immediately after transfection with the plasmid expressing FoxO1 (or Mock). Pyroptotic cell death was assessed using flow cytometry for propidium iodide (PI) and active caspase-1.



Supplementary Figure 9

Supplementary Figure 9. The effect of clodronate treatment on TUNEL-positive hepatocytes

C57BL/6 mice were first intravenously injected with 10 mL/kg body weight of clodronate (or vehicle), and after a week, they were subjected to Lieber-DeCarli alcohol liquid diet (or control diet) with additional injections of clodronate (5 mL/kg body weight, i.p., every 4 days) for 5 weeks as described in Figure 6A. TUNEL staining was carried out for the liver sections. Representative data were shown (n=3 each).

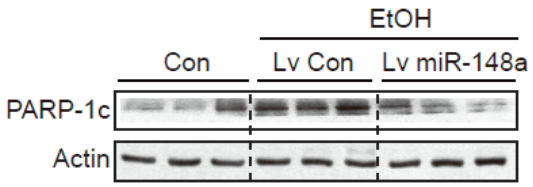


Supplementary Figure 10

Supplementary Figure 10. The effects of hepatocyte-specific delivery of miR-148a on serum IL-1β and TUNEL-positivity in the liver

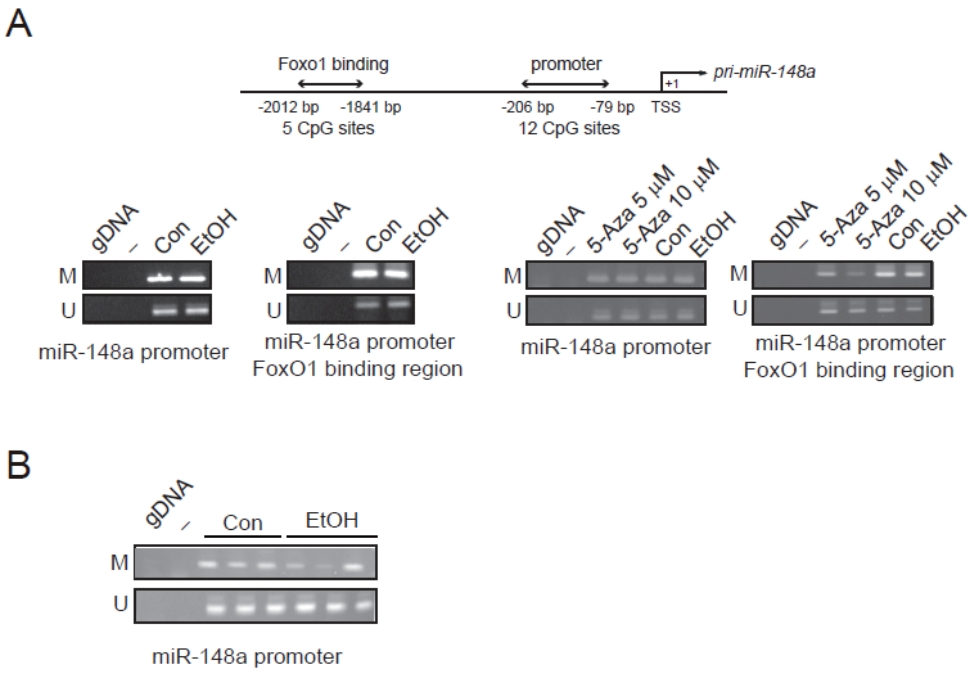
(A) ELISA assays for serum IL-1β. After hepatocyte-specific lentiviral delivery of miR-148a (or Lv-Con), the mice were subjected to Lieber-DeCarli diet or control diet as described in Figure 7A. Data represents the mean ± SEM (n=4-6/group, significantly different as compared to each control, *$p<0.05$, **$p<0.01$). Statistical comparisons among multiple groups were done by one-way ANOVA with Bonferroni correction.

(B) TUNEL staining of the liver sections. Representative liver sections were shown (n=3 each). Scale bar, 100 μm



Supplementary Figure 11

Supplementary Figure 11. Immunoblotting for cleaved PARP-1 (PARP-1c) in the liver samples used in Figure 7B



Supplementary Figure 12

Supplementary Figure 12. The methylation status of miR-148a gene promoter after alcohol challenge

(A) The promoter region and the predicted FoxO1 binding region with CpG sites in the miR-148a gene analyzed for methylation specific PCR (MSP) assays (upper). TSS, transcription start site. MSP assays were done for the miR-148a gene promoter or the FoxO1 binding region (lower). AML-12 cells were treated with 100 mM ethanol or vehicle (medium, Con) for 48 h (left). AML-12 cells were also treated with 5 or 10 μM 5-aza-2′-deoxycytidine (5-Aza) or vehicle (dimethylsulfoxide) for 72 h; or with 100 mM ethanol for 48 h (right). The media with treatment agent were refreshed every 24 h.

(B) MSP assay for miR-148a gene promoter in liver tissue. Mice were fed on either control diet or Lieber-DeCarli alcohol diet for 6 weeks (n=3 each) as described in Figure 1C. M, methylated; and U, unmethylated

Supplementary Table 1. Representative miRNAs dysregulated in liver diseases

Liver diseases	Expression pattern		References
	Increase	Decrease	
Viral hepatitis			
HBV	miR-23a, miR-146a, miR-181a, miR-196b	miR-17, miR-338-3p , miR-378	1, 2
HCV	miR-16, miR-21, miR-34a, miR-122, miR-155	miR-199a, miR-200, miR-122	3, 4, 5
NAFLD	miR-21, miR-34a	miR-122, miR-126	6
ALD	miR-217, miR-182, miR-155	miR-122	7, 8, 9, 10
Fibrosis	miR-34a, miR-199a miR-221	miR-150, miR-194, miR-200	3, 5, 11
HCC	miR-21, miR-135a, miR-146a, miR-151, miR-221, miR-222	miR-29, miR-122, miR-148a, miR-192, miR-194, miR-199a	5, 12, 13, 14

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; NAFLD, non-alcoholic fatty liver disease; ALD, alcoholic liver disease; and HCC, hepatocellular carcinoma

Supplementary Materials and Methods

Materials

Anti-FoxO1, anti-IL-1β, and anti-PARP-1 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies directed against TXNIP, NLRP3, and ASC were provided from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-caspase-1, anti-CYP2E1, and anti-FoxO3a antibodies were purchased from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were obtained from Zymed Laboratories (San Francisco, CA). Anti-β-actin antibody and other reagents were supplied from Sigma (St. Louis, MO, USA). Alexa Fluor 488–conjugated anti-mouse IgG, Alexa Fluor 555–conjugated anti-rabbit IgG, fluorescein–conjugated anti-rabbit IgG, and Alexa Fluor 568–conjugated anti-mouse IgG were obtained from Invitrogen (Carlsbad, CA, USA).

Human samples

Liver samples were randomly selected from a cohort of consecutive patients with clinical, analytical, and histological features of AH admitted to the Liver Unit of the Hospital Clinic of Barcelona from 2011 to 2015. Inclusion and exclusion criteria and baseline clinical characteristics of AH patients and healthy controls have been described previously.[15] For miRNA microarray analysis, thirteen AH patient samples were used; the clinical and biochemical parameters of patients with AH were shown in Ref 15. Informed consent was obtained from all patients, according to the ethical guidelines of the 1975 Declaration of Helsinki and approval by the Ethics Committee of the Hospital Clinic of Barcelona.

Non-tumorous liver tissues adjacent to liver cancer collected during the period between 2006 and 2009 were supplied from the Asan Medical Center (Seoul, Korea) after review and approval by institutional review board (#2012-0133) as previously reported.[14] Informed consents from the patients were obtained before operations. To assess TXNIP levels in the liver samples from patients, ten liver portal fibrosis samples with different etiologies were selected for immunoblotting. Histological assessment of TXNIP was performed in normal liver tissue obtained from surgical resections of liver metastasis and in samples of non-tumor liver tissue obtained from alcoholic cirrhotic patients. Informed

consent was obtained from all patients, according to the Ethics Committee of the Hospital Clinic of Barcelona.

Reverse transcription and quantitative PCR assays

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), and was reverse-transcribed to obtain cDNA using the miScript Reverse Transcription kit (Qiagen, Hilden, Germany). For assays of mRNA, cDNA was generated using the PCR Master kit (Roche, Mannheim, Germany). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was carried out using ABI StepOne plus Real-Time PCR System and 48-well optical reaction plates (Applied Biosystems, Foster City, CA, USA). The following primer sequences were used: mouse β–actin, 5'-CTGAGAGGGAAATCGTGCGT -3' and 5'- TGTTGGCATAGAGGTCTTTA -3'; 18s RNA, 5'-GTAACCCGTTGAACCCCATT -3' and 5'- CCATCCAATCGGTAGTAGCG -3'; mouse FoxO1, 5'-CTGGGTGTCAGGCTAAGAGT -3' and 5'- GGGGTGAAGGGCATCTTT -3'; mouse TXNIP, 5'-TCAAGGGCCCCTGGGAACATC -3' and 5'- GACACTGGTGCCATTAAGTCAG -3'; and mouse pri mir-148a, 5'- CCTGCAGGACGAAACTTCCA -3' and 5'- AAAGGA AAGGTGCAACGACG -3'. For qRT-PCR assays for miRNA, cDNA was generated from equal amounts of total RNA per sample (1 μg) using the miScript Reverse Transcription kit (Qiagen GmbH, Hilden, Germany). The reaction mixture containing reverse transcription product, 2×QuantiTect SYBR Green PCR Master Mix, 10×miScript Universal Primer, and primers was incubated at 95°C for 15 min, followed by 40 amplification cycles of 94°C for 10 s, 55°C for 30 s, and 70°C for 30 s. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. Transcripts of U6 small RNA were quantified using the Hs_RNU6B_2 miScript Primer Assay (Qiagen, Hilden, Germany) for normalization of miRNA levels.

Heatmap data were extracted from the miRNA array database (GSE59492) available in public domain.[1] To validate miRNA expression for human subjects, qRT-PCR was performed on liver samples from 12 AH patients and 6 healthy individuals. MiRNA quantification was done using the commercial kit Mir-X miRNA First-Strand Synthesis Kit according to the manufacturer's instructions (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). Data were normalized using U6 snoRNA as

endogenous control. To assess gene expression in human samples, cDNA was obtained with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). qRT-PCRs were accomplished with Taqman Human Assays using ABI 7900 HT cycler (Life Technologies, Grand Island, NY). All primers used to determine GENE expression were Taqman probes (Thermo Fisher Scientific, Waltham, MA): 18sRNA, Hs99999901_s1; human FoxO1, Hs01054576_m1; and human TXNIP, Hs01006900_g1.

Animal treatments

Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Use and Care Committee at Seoul National University. Male C57BL/6 mice at 6-8 weeks of age were fed the Lieber-DeCarli diet (Dyets Inc.) with 5% (vol/vol) ethanol (36% ethanol derived calories) or the isocaloric liquid diet *ad libitum* for 4-6 weeks. For binge alcohol model, mice were administered with ethanol (5 g/kg body weight) diluted (50:50; vol/vol) in water twice daily by gastric intubation for 7 consecutive days. Control mice received tap water. For *in vivo* miRNA delivery experiment, mice were injected with lentiviruses that express pre-miR-148a (or control) through tail vein ($2x10^7$ TU each in 200 μL phosphate-buffered saline per mouse). A lentivirus transcribing the miR-148a precursor (miR-148a), and the corresponding control were produced by Seolin Biosciences (Seoul, Korea). For lentivirus encoding miR-148a, the mouse albumin enhancer/promoter (NB) construct was kindly provided by Dr. Richard D. Palmiter (University of Washington). Seven days after the injection, the mice were fed on a Lieber-DeCarli diet *ad libitum* for 4 weeks. Blood and liver samples taken from mice were biochemically and histopathologically analyzed. The study protocol was approved by the Institutional Biosafety Committee of Seoul National University (SNUIBC-R151119-1).

Cell culture

AML-12 cells (a mouse hepatocyte-derived cell line) and HepG2 cells (a human hepatocyte-derived cell line) were purchased from American Type Culture Collection (Manassas, VA, USA). Hepatocytes were isolated from C57BL/6 mice, as previously described.[16] Briefly, under anesthesia with Zoletil, livers were perfused with Ca^{2+}-free Hank's buffered salt solution (Invitrogen, Carlsbad, CA, USA) for

10 min, followed by continuous perfusion with a 0.1% w/v collagenase (Sigma, Type I). The whole liver was removed, and minced in PBS. Cell suspension was filtered through the cell strainer (70 μm), and purified with Percoll. Primary hepatocytes were harvested into collagen-coated plates ($5x10^5$ cells/well) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 units/mL penicillin, and 50 μg/mL streptomycin. Six hours later, the cells were treated with vehicle or 100 mM ethanol for 48 h. HepG2 cells were maintained in the DMEM containing 10% FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin. AML-12 cells were cultured in the DMEM/F-12 containing 10% FBS, insulin-transferrin-selenium X, dexamethasone (40 ng/mL; Sigma), and the antibiotics. For alcohol models, AML-12 cells or primary hepatocytes were exposed to 100 mM ethanol (freshly diluted in media) for most of assays, or 200 mM for pyroptosis assays, for indicated times. To prevent ethanol evaporation during exposure, each culture dish was tightly wrapped with Parafilm.

To assess methylation status of miR-148a promoter, AML-12 cells were treated with 5 or 10 μM 5-aza-2'-deoxycytidine (5-Aza; Sigma) or dimethylsulfoxide (as vehicle) for 72 h, or with ethanol for 48 h. The media containing treatment agent were refreshed every 24 h.

Analysis of integrative network

Gene expression data were obtained from the Gene Expression Omnibus (GSE28619) available in the public domain. Unsupervised K-means clustering method was performed using Euclidean distance measure (K=4), and the down-regulated genes involved in one cluster were selected and clustered by gene ontology using DAVID 6.7 software.[17,18] Interactions of the genes in the apoptosis-related cluster were analyzed using STRING v9.1 database.[19]

Immunoblot analysis

Proteins were resolved by 7.5% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked with 5% non-fat dried milk in TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.4) for 1 h and incubated overnight with each primary antibody at 4°C. After washing with TBST buffer, membranes were incubated with secondary antibodies for 1 h at room temperature. The protein bands were visualized

using an enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK). Equal loading of samples was verified by immunoblotting for β-actin. Band intensities were quantified using Photoshop CS5 (Adobe Systems, San Jose, CA, USA).

Chromatin immunoprecipitation (ChIP) assays

HepG2 cells were transfected with a construct encoding FoxO1, and then formaldehyde was added to the cells to a final concentration of 1% for cross-linking of chromatin. The chromatin immunoprecipitation assay was performed according to the EZ-ChIP assay kit protocol (Upstate Biotechnology, Lake Placid, NY, USA). PCR was done using the primers flanking the FoxO1 regions located in the promoter region of human *MIR148A* gene (FoxO1-RE 1/2: sense, 5'-GGTTCGTCTGCTACCCAACA -3' and antisense, 5'- GAGCCAGCCTGCTGATGTAA -3'; FoxO1-RE 3: sense, 5'- ACAACAGAACAGGCAGCAGA -3' and antisense, 5'- CCCCATTTCCCCCTATGGAA -3'; or an irrelevant region of the gene (sense, 5'- AAATTGATTCTACGTGCCCG - 3' and antisense, 5'-GTCCCCTTCTTTTTCCTTGG -3'). One tenth of cross-linked lysates served as the input control.

Small interfering RNA (siRNA) knockdown

Scrambled siRNA (control) and siRNAs of FoxO1 and TXNIP were supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with each siRNA (100 pmole) using FuGENE HD (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Transient transfection and reporter gene assays

The plasmid overexpressing FoxO1 was supplied from Addgene (Cambridge, MA, USA). Cells were plated in six-well plates overnight, serum-starved for 3 h, and transiently transfected with the plasmid in the presence of FuGENE HD Reagent (Promega, Madison, WI, USA). The transfected cells were then incubated in Eagle's minimum essential medium containing 1% fetal bovine serum for 18 h. For reporter gene assays, the region containing –1,800 bp to +1,097 bp of the human *MIR148A* gene was cloned into the pGL3 luciferase vector. A mutation of FoxO1-RE3 was done by replacing the sequence of FoxO1 binding element from 5'- ATAAAC**AA**ACA -3' to 5'- ATAAAC**GC**CACA -3' (bolds indicate mutations).

Cells were transfected with FoxO1 siRNA (siFoxO1) for 48 h in the presence of FuGENE HD reagent, and luciferase activity was measured by adding luciferase assay reagent (Promega, Madison, WI, USA).

Transfection of miRNA mimic or antisense oligonucleotide

Synthetic miRNA duplexes were synthesized and transfected, as previously described.[2] Briefly, the cells in each well (6-well plates) were transiently transfected with 100 pmoles of miR-148a mimic or control mimic (GenePharma, China), or 100 pmoles of 2'-O-methyl miR-148a antisense oligonucleotide (ASO) or respective control ASO using FuGENE HD Reagent (Promega, Madison, WI, USA). In case of primary hepatocytes, Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used.

3'UTR luciferase assays

The miRNA 3'UTR target clone (Luc-TXNIP-3'UTR, binding site sequence; TGCACTG, GCACTGA) was purchased from GeneCopoeia (Rockville, MD, USA), which contains renilla luciferase as internal control fused downstream to firefly luciferase. The cells were co-transfected with Luc-TXNIP-3'UTR luciferase or control vector, and miR-148a mimic (or inhibitor) or its respective control using FuGENE® HD Reagent (Promega, Madison, WI, USA). At 48 h after transfection, firefly and renilla luciferase activities were measured using Luc-Pair miR Luciferase Assay (GeneCopoeia) according to the manufacturer's protocols. The two target sites within TXNIP 3'UTR reporter were mutated to generate a mutant reporter construct (5'-T**CGTGA**G-3' and 5'-G**GTGAC**A-3', bolds indicate mutations), which was used as a negative control.

Immunocytochemistry

AML-12 cells were grown on a coverslip and were fixed in a 4% paraformaldehyde solution followed by permeabilization with 0.3% Triton X-100 (Sigma Aldrich). After washing with PBS, the cell samples were immunostained with antibodies directed against TXNIP and NLRP3. Fluorescein–conjugated anti-rabbit IgG and Alexa Fluor 568–conjugated anti-mouse IgG (1:500, Invitrogen, Carlsbad, CA, USA) were used as secondary antibodies. After incubation, the samples were cover-slipped with

mounting media containing Hoechst 33342 NucBlue® Live ReadyProbes TM Reagent (Invitrogen, Carlsbad, CA, USA).

Flow cytometric analysis

Pyroptosis was analyzed by the FAM-FLICA in vitro Caspase-1 Detection Kit (ImmunoChemistry Technologie, Bloomington, MN, USA) according to the manufacturer's instructions. Briefly, the transfected cells were harvested by trypsinization. After washing with PBS, the cells were stained with 10 μL FAM-FLICA and 2 μg/mL PI in cell culture media containing 20% FBS. In each analysis, 20,000 gated events were recorded. For the analysis of TXNIP and FoxO1, the transfected cells were harvested by trypsinization. After washing with PBS, the cells were stained for TXNIP (1:100) and FoxO1 (1:200) with 0.3% Triton X-100 permeabilization. Alexa Fluor 488–conjugated anti-mouse IgG and Alexa Fluor 555–conjugated anti-rabbit IgG (1:200, Invitrogen, Carlsbad, CA, USA) were used as secondary antibodies. In each analysis, 5,000 gated events were recorded. The fluorescence intensity in the cells was assessed using FACS Calibur II flow cytometer and the CellQuest software (BD Biosciences, San Jose, CA, USA).

Histological analysis

The liver tissues were subjected to hematoxylin and eosin (H&E) and immunohistochemistry (IHC). Liver specimens were fixed in 10% formalin, embedded in paraffin, cut into 4 μm thick sections, and mounted on slides. Tissue sections were immunostained with antibodies directed against F4/80, TXNIP, and caspase-1 for 2 h, followed by polink-2 plus polymer HRP detection (GBI, Bothell, WA, USA). To validate the specificities of anti-TXNIP and anti-caspase-1 antibodies, liver tissues of TXNIP KO mice and small intestine tissues of caspase-1 KO mice were used, respectively (data not shown). The individual tissue sections were kindly provided by Dr. Inpyo Choi (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea) and Dr. So-Youn Woo (Ewha womans University, Seoul, Korea). For immunohistochemical assays of alcoholic cirrhotic patients, tissue sections were immunostained with anti-TXNIP antibody (Novus Biologicals) and then incubated with polink-2 plus polymer HRP detection. Densitometry analysis of TXNIP positive immunostained images was

performed using ImageJ software (NIH). TUNEL assays were carried out using the in situ S7100 ApopTAG® apoptosis detection kit from Millipore (Temecula, CA), and the DeadEnd Colorimetric TUNEL System from Promega (Madison, WI), according to the manufacturer's instructions.

Serum parameter

Serum IL-1β contents were measured using IL-1β ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Bisulfite conversion of genomic DNA and methylation specific PCR (MSP)

Genomic DNA was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer). The methylation status of the miR-148a promoter region was determined by methylation-specific PCR (MSP) using bisulfite-modified DNA. Bisulfite conversion of genomic DNA of each specimen was performed using EZ DNA Methylation™ Kit (Zymo Research). The mouse primers were designed for MSP according to the results of bisulfite-modified sequencing. The methylation-specific primer set and product size were shown as below. The PCRs were conducted under the following thermocycling conditions: 2 minutes at 94°C; 45 cycles of 30 seconds at 94°C, 30 seconds at 56~58°C (depends on regions), and 30 seconds at 72°C; and a final incubation at 72°C for 7 minutes. Controls without DNA and genomic DNA without bisulfite treatment were used for each set of PCRs. Aliquots of 10 μL of the total 20 μL PCR mixture were loaded onto 2% agarose gel, stained with ethidium bromide, and directly visualized under ultraviolet illumination. The following primer sequences were used: miR-148a-promoter M forward, 5′-GAATTTGTTGATTTGATACGAGTC-3′, reverse, 5′-AATTTTAAATTTAAAAACGACCTACG-3′; or miR-148a promoter FoxO1 binding region M forward, 5′-TATGAATGGATAATAGGGTATTTGC-3′, reverse, 5′-ATCAATCAAAAAATAACAATACGTT-3′, whereas the others were specific for the unmethylated sequence, miR-148a promoter U forward, 5′-GGAATTTGTTGATTTGATATGAGTT -3′, reverse, 5′- TTTAAATTTAAAAACAACCTACACT -3′; or miR-148a promoter FoxO1 binding region U forward, 5′-TATGAATGGATAATAGGGTATTTGTGA-3′, reverse, 5′-ATTAAAATCAATCAAAAAATAACAATACAT-3′. The primers used in the present study specifically

detected the promoter sequence of the miR148a gene. M and U are the PCR products of the methylated and unmethylated alleles, respectively.

Supplementary References

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