Table S1, related to Figure 3: STAT3 binding sites in microRNAs

Table S1. STAT3 binding sites in microRNAs

MicroRNA	Binding Sites (hg18)	PhylCRM Score
miR-24	chr19:13,808,863-70 (CACTTCCT)	198.97
miR-629	chr15: 68,176,873-80 (CCCTTCCC)	159.72

Table S2, related to Figure 4: HNF4α binding sites in microRNAs

Table S2. HNF4a binding sites in microRNAs

MicroRNA	Binding Sites (hg18)	PhylCRM Score
miR-7-1	chr9: 85780032-44	271.819
miR-124	chr8: 65443748-60	206.073
miR-9-2	chr5: 88007516-28	188.182
miR-29a	chr7: 130235232-44	174.358
miR-10a	chr17: 44016497-6509	162.068
miR-601	chr9: 125730805-17	150.576
miR-190b	chr1: 152476121-33	137.471
miR-568	chr3: 115578485-97	100.705

Experimental Procedures

Cell Culture

Human non-transformed NeHepLxHT immortalized hepatocytes (IMH1) were purchased from ATCC (cat no. CRL-4020). This is a diploid human cell line of male origin which was developed from hepatocytes by transduction with a retroviral expression vector (pLXSN) containing the hTERT gene. IMH1 cells were maintained in DMEM:F12 medium supplemented with 10% heat-inactivated FBS, 0.1 microliter/ ml dexamethasone from HMM Hepatocyte Medium SingleQuot (Lonza/ Clonetics Catalog # CC-4192), 0.11 microliter/ ml insulin from HMM Hepatocyte Medium SingleQuot (Lonza/ Clonetics Catalog # CC-4192), 50ug/ml G-418 and 10 units/ml penicillin, and 100 µg/ml streptomycin.

The immortalized hepatocyte cell line (designated Fa2N-4) was purchased from Xenotech LLC (cat. no. IFH15). The cell line (IMH2) was isolated from normal human hepatocytes of female origin and transformed with the SV40 virus large T-antigen. Fa2N-4 hepatocytes attach to collagen (Purecol/MCDI solution, Purecol from Fisher Scientific cat no. 50-360-230/MCDI from Sigma C10640-2) and adopt the well-defined, cubical shape that is characteristic of human primary hepatocytes. The cells were thawed and plated using the Multi-Function Enhancing (MFE) Plating medium F (Xenotech LLC, cat no. K4005) supplemented with 5 units/ml penicillin, 50 µg/ml streptomycin, and 10% component B (Xenotech LLC). The MFE plating medium F was replaced with serum-free MFE Support medium F (Xenotech LLC, cat no. K4105), supplemented with 5 units/ml penicillin, and 50 µg/ml streptomycin, 4 hours after plating. The next day the medium was replaced by MFE Support medium F supplemented with MFE supplement A (Xenotech LLC) and the cells were subjected to the appropriate analysis.

Human liver cancer cell lines HepG2 were maintained in DMEM medium (Gibco) supplemented with 10% FBS and 10 units/ml penicillin, and 100 µg/ml streptomycin. SNU-449 cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS and 10 units/ml penicillin, and 100 µg/ml streptomycin.

Reagents

SiRNAs: The following siRNAs were used in this study: siRNA negative control (siNC, cat no. AM4611, Ambion Inc) and two different siRNAs against HNF4α (siHNF4α#1, cat no. s6696, Ambion Inc) and (siHNF4α#2, cat no. s6698, Ambion Inc).

ShRNAs: shRNA control (pGFP-V-RS, cat. no TR30007, Origene Inc), shRNA against HNF4α (shHNF4α#1, cat.no TG320382, Origene Inc).

MicroRNAs: miR-24 (cat no. C-300497-03-0005, Dharmacon Inc), as-miR-24 (cat no. IH-300497-03-0005, Dharmacon Inc), miR-629 (cat no. C301129-01-0005, Dharmacon Inc), as-miR-629 (cat no. C301129-02-0005, Dharmacon Inc), miR-124 (cat no. C-300593-05-0005, Dharmacon Inc), as-miR-124 (cat no. IH-300593-06-005, Dharmacon Inc), miR NC (cat no. CN-001000-01-05, Dharmacon Inc) and as-miR NC (cat no. IN-001005-01-05, Dharmacon Inc)

Antibodies: HNF4α (sc-6556, Santa Cruz Biotech Inc), STAT3 (9139, Cell signaling), pSTAT3 (9138, Cell Signaling), IL6R (sc-13947, Santa Cruz Biotech Inc) and b-actin (4970, Santa Cruz Biotech Inc).

MicroRNA Library Screen

A microRNA library, consisting of 317 microRNA mimics and 2 microRNA negative control mimics (100nM) (Dharmacon Inc) was transfected in HepG2 cells plated in 96-well plates (three replicates). The transfection dose of 100nM for the microRNA mimics was detected through control experiments performed to identify the maximum dose without any cytotoxic effects. 24h post-transfection, the cells were transfected with a firefly luciferase vector harboring the 3'UTR of HNF4α (cat no. HmiT008908-MT01, Genecopoeia Inc) for 24h and the luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, WI, USA). MicroRNAs that inhibited >75% the luciferase activity of HNF4α in all three replicates were considered as positive hits. Comparison of the three replicates suggested high reproducibility of the screen. Furthermore, we performed a secondary screen using a 3'UTR vector that does not have any binding sites for the positive hits microRNAs identified from the primary screen, showing the specificity of our findings. The positive hits identified in the primary screen were validated in secondary screens in HepG2 and Hep3B cells (Figure 1C). Specifically 100nM of these microRNA mimics were transfected in HepG2 and Hep3B cells plated in

6-well plates, 24h post-transfection, HepG2 and Hep3B cells were transfected with the firefly luciferase vector harboring the 3'UTR of HNF4α for 24h and the luciferase activity was measured using the Dual Luciferase Reporter Assay System. The identification of these hits in both HepG2 and Hep3B cell lines, suggest that the findings of the primary screen are not cell line-dependent.

Colony Formation Assay

IMH1, IMH2, HepG2, Hep3B, and SNU-449 cells were transfected with shRNAs, siRNAs or microRNAs for 48h. Then, triplicate samples of 10^5 cells from each cell line were mixed 4:1 (v/v) with 2.0% agarose in growth medium for a final concentration of 0.4% agarose. The cell mixture was plated on top of a solidified layer of 0.8% agarose in growth medium. Cells were fed every 6 to 7 days with growth medium containing 0.4% agarose. The number of colonies was counted after 20 days. The experiment was repeated thrice and the statistical significance was calculated using Student's t test.

Invasion Assays

We performed invasion assays in IMH1, IMH2, HepG2, Hep3B and SNU-449 cells under different transfection conditions with siRNAs or microRNAs for 24h. Invasion of matrigel has been conducted by using standardized conditions with BDBioCoat growth factor reduced MATRIGEL invasion chambers (PharMingen). Assays were conducted according to manufacturer's protocol, by using 10% FBS as chemoattractant. Non-invading cells on the top side of the membrane were removed while invading cells were fixed and stained with 4′-6-diamidino-2-phenylindole (DAPI, Vector Laboratories Inc.), 16h post seeding. In all assays, 10 fields per insert were scored and SD was measured. The experiment was repeated thrice and the statistical significance was calculated using Student's t test.

Real-time PCR analysis

RNA purified from IMH1, IMH2, HepG2, Hep3B and SNU-449 cells under different transfection conditions with siRNAs or microRNAs was reverse-transcribed to form cDNA, which was subjected to SYBR Green based real-time PCR analysis. Primers used for actin forward: 5'-CCTGTACGCCAACACAGTGC-3' and reverse 5'-ATACTCCTG CTTGCTGATCC-3'; HNF4α forward: 5'- TGTCCCGACAGATCACCTC -3' and reverse 5'- CACTCAACGAGAACCAGCAG -3'; ApoCIII forward: 5'-GGGTACTCCTTGTTGTTGC -3' and reverse: 5'- AAATCCCAGAA CTCAGAGAAC -3'; ALDOB forward: 5'- AGGAGGACTCTTCTCTCCCAA-3' and reverse: 5'-GATTCATCTGCAGCCAGGAT-3'; CYP1A2 forward: 5'-CTGGCCTCTG CCATCTTCTG-3' and reverse: 5'- TTAGCCTCCTTGCTCACATGC-3'; CYP7A1 forward: 5'-CAGTGCCTCCCTCAACATCC-3' and reverse: 5'- GACATATTGTAGCTCCC GATCC-3'; PEPCK forward: 5'- AGCTCGGTCGCTGGATGTCAGAG-3' and reverse 5'-GTAGGGTGAATCCGTCAGCTCGATG-3'; G6P forward: 5'- GGCTCCATGACTGTGG GATC-3' and reverse: 5'- TTCAGCTGCACAGCCCAGAA-3'; IL6R forward: 5'-TGCCAGTATTCCCAGGAGTC-3' and reverse: 5'- GGCAGTGACTGTGATGTTGG-3'.

MicroRNA real-time PCR analysis.

MicroRNA expression levels were tested using the Exiqon PCR Primer Sets, according to the manufacturer's instructions (Exiqon Inc, Denmark). Specifically we tested the expression levels of miR-24 (cat no. 204260, Exiqon), miR-629 (cat no. 204370) and miR-124 (cat no. 204319). U6 expression was used as an internal control. The expression levels of these microRNAs in liver cancer cell lines were normalized to the microRNA levels of the immortalized hepatocytes (IMH1). The experiments have been performed in triplicate and data are presented as mean ± SD.

Western Blot analysis

Protein samples were subjected to SDS PAGE and transferred to polyvinylidene difluoride membranes in 25 mM Tris, 192 mM glycine. Membranes were blocked with 5% nonfat dry milk in PBS, 0.05% Tween-20 and probed with antibodies (1:1000) followed by corresponding horseradish peroxidase-labeled secondary antibodies

(1:1000). Blots were developed with ECL reagent (PerkinElmer Life Sciences, Waltham, MA) and exposed in Eastman Kodak Co. 440 Image Station.

Cell Cycle analysis

Cells were trypsinized, pelleted at 1,000 x g and 4°C for 5 min, and lysed in lysis-staining buffer (3.4 mM sodium citrate, 10 mM NaCl, 0.1% Nonidet P-40, 75 µM ethidium bromide [EtBr]) (1 ml/10^6 cells on ice). The fluorescence intensity of cell nuclei was measured by fluorescence-activated cell sorter (FACS) analysis.

BrdU staining

DNA synthesis was determined using the 5-Bromo-2'-deoxy-Uridine (BrdU) Labeling and Detection Kit II according to manufacturer's instructions (11299964001, Roche).

Caspase luciferase activity. Relative 3/7 caspase activity was measured using the Caspase-Glo 3/7 Assay according to manufacturer's instructions (G8091, Promega). Measurements were normalized to the respective cell numbers which were determined using the CellTiter-Glo Luminescent Cell Viability Assay (G7571, Promega). Results are expressed as the percent of the ratio of caspase activity/cell number compared to the siControl-treated cells (Figure S18).

Bio-Plex Phospho-STAT3 (Tyr 705) ELISA Assay. This sandwich ELISA assay (cat**.** no 171-V22552, Bio-rad) assessed the phosphorylation status of tyrosine 705 of STAT3 protein in ELISA in HepG2, Hep3B and SNU-449 cells treated 100nM siRNA NC, siRNA against HNF4α (siHNF4α#1), miR-24 and/or miR-629, as-miR-NC, as-miR-124 for 24h. The data were analyzed in a Bio-plex FlexMap3D analyzer using the Bio-plex manager software.

Cleaved Caspase-3 ELISA assay. PathScan Cleaved Caspase-3 (Asp175) Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of cleaved caspase-3 protein (Cell Signaling Inc, cat no.

7190). A total Caspase-3 Antibody has been coated onto the microwells. After incubation with cell lysates, the caspase-3 (cleaved and uncleaved) protein is captured by the coated antibody. Following extensive washing, A biotinylated Cleaved Caspase-3 Antibody is added to detect the captured cleaved caspase-3 protein. HRP-linked streptavidin is then used to recognize the bound detection antibody. HRP substrate, TMB, is added to develop color. The magnitude of optical density for this developed color is proportional to the quantity of cleaved caspase-3 protein. This assay was used to measure the levels of cleaved caspase-3 in DEN-treated mice which were administered with miR-NC or miR-124 (data presented in figure 6D).

Cleaved PARP ELISA assay. PathScan Cleaved PARP (Asp214) Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of cleaved PARP (Asp214) protein (Cell Signaling Inc, cat no. 7262). A cleaved PARP (Asp214) Mouse mAb* has been coated onto the microwells. After incubation with cell lysates, cleaved PARP (Asp214) protein is captured by the coated antibody. Following extensive washing, PARP Rabbit mAb is added to detect the captured cleaved PARP protein. Anti-rabbit IgG, HRP-linked Antibody is then used to recognize the bound detection antibody. HRP substrate, TMB, is added to develop color. The magnitude of the absorbance for this developed color is proportional to the quantity of cleaved PARP (Asp214) protein. This assay was used to measure the levels of cleaved PARP in DEN-treated mice which were administered with miR-NC or miR-124 (data presented in figure 6D).

IL6 ELISA Assay. This assay (R&D Systems, cat. no D6050) Systems employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody , for IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-6 bound in the initial step. The color development is stopped and the intensity of the color is measured.

IL6R ELISA assay. The Sino Biological ELISA kit (cat no. SEK10398) is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). It utilizes a monoclonal antibody specific for IL6R coated on a 96-well plate. Standards and samples are added to the wells, and any IL6R present binds to the immobilized antibody. The wells are washed and a biotinylated rabbit anti-IL6R polyclonal antibody is then added, producing an antibody-antigen-antibody “sandwich”. To produces color in proportion to the amount of IL6R / CD126 present in the sample strepavidin-HRP and TMB substrate solution are loaded. The absorbances of the microwell are read at 450 nm.

Both IL6 and IL6R assays were used to measure soluble IL6R levels in untreated, 1nM of siRNA NC or 1nM of siRNA against HNF4a (siHNF4a#1) in HepG2, Hep3B and SNU-449 cells, 24h post transfection (data are shown in figure S16A, B). Furthermore, we measured IL6R levels soluble in IMH1 cells transfected with 1nM siRNA NC or siHNF4a#1, using this assay (data shown in figure S17H). In addition, we measured IL6 and IL6R levels in human hepatocellular tissues (data shown in figure 7E).

Serum assays

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin and urea levels in mice were determined with commercially available kits (Sigma).

Luciferase assays

Untreated or miR-NC, miR-124, as-miR NC, as-miR-124 (100nM) treated HepG2 cells were transfected with a firefly luciferase reporter gene construct containing the 3’UTR of IL6R (HmiT009672-MT01, Genecopoeia Inc) Cell extracts were prepared 24h after transfection of the luciferase vector, and the luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, WI, USA). In addition, HepG2 cells were transfected with a firefly luciferase reporter gene construct containing the miR-124 regulatory area (wild type or deletion mutant in the HNF4α binding site) and 12 and 24h post treatment with IL6 (20ng/ml) were lysed and the luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

MicroRNA Target Prediction Analysis

The miRNA database TargetScan version 5.1 (http://www.targetscan.org/index.html) was used to identify potential miR-124, miR-24 and miR-629 targets.

Identification of transcription factor sites in microRNA regulatory areas

The Lever and PhylCRM algorithms were developed previously and are described in a separate paper (Warner, J. et al. Systematic identification of mammalian regulatory motifs' target genes and their functions. *Nature Methods, 2008 Apr; 5(4):347-53.*) In order to identify STAT3 and HNF4α binding motifs in an area of 5kb upstream and 2kb downstream of microRNAs we used three selection criteria: 1) First we examined the presence of STAT3 and HNF4α binding motifs using Lever algorithm. Lever assesses whether the motifs are enriched in cis-regulatory modules (CRMs), in the noncoding sequences surrounding the genes; 2) For the identified binding sites we incorporated phylogenetic information from 12 different mammal species (mouse, rat, human, rabbit, chimp, macaque, cow, dog, armadillo, tenrec, opossum and elephant) and selected only the binding sites with high conservation scores (a conserved motif is considered one with a score higher than 100) by using PhylCRM algorithm (for more details see Warner et al., 2008). 3) Then, we mapped the conserved binding sites in the regions of interest, as well as visually inspecting the nucleosome occupancy in the conserved binding sites shown as part of the UCSC genome browser track.

STAT3 binding sites: The predicted and conserved STAT3 binding motif in miR-24 was located in chromosome 19:13,808,863-13,808,870 and had a conservation score of 198.97, the predicted and conserved STAT3 binding motif in miR-629 was located in chromosome 15: 68,176,873-68,176,880 and had a conservation score of 159.72.

HNF4α binding sites: The chromosomal coordinates and conservation scores for the predicited HNF4α binding sites in microRNA regulatory areas are shown in Table S1.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was carried out as described previously (Iliopoulos et al., Mol Cell, 2010). Briefly, the chromatin fragments, derived from untreated and IL6-treated (6, 12, 24h) SNU-449 cells, were immunoprecipitated with 6ug of antibody against STAT3. DNA extraction was performed using Qiagen Purification Kit. Real-time PCR

analysis was performed for STAT3 binding sites in microRNA regulatory areas using the following primers: miR-24: forward 5'-ATGGGGAGAGGAAGCCAAG-3' and reverse 5'-CTAAGCCCTGGCCACTGA-3' (PCR product: 150bp); miR-629: forward 5'-CCCCCTCGGAGAGGAGAG-3' and reverse 5'-GTGCCCGCTGGACTTAGG-3' (PCR product: 150). In addition, chromatin immunoprecipitation was performed in HepG2 and SNU-449 cells by using 9μg of antibody against HNF4α. Real-time PCR analysis was performed for HNF4 binding sites in microRNA regulatory areas using the following primers: miR-7-1: forward 5'- TTGATTTACAATGCGGCAAA-3' and reverse 5'-TCCCCTTTAGACGGTGTATTG-3' (PCR product: 169bp); miR-124: forward 5'-AGAGGAAGAGACCGGGAGTG-3' and reverse 5'- TTGAGAAGCCCTG GACAGAT-3' (PCR product: 152bp).

Mouse experiments

$5x10^6$ IMH1 cells transfected with 100nM siRNA NC or siHNF4α#1 or siHNF4α#2 or microRNA negative control (miR NC) or miR-24 and/or miR-629 for 48h were injected subcutaneously in the right flank of NOD-SCID mice (five mice/group). Tumor growth was monitored every five days for a total period of 30-55 days. Tumor volumes were calculated by the equation $V(mm^3)=axb^2/2$, where a is the largest diameter and b is the perpendicular diameter. In addition, $5x10^6$ SNU-449 cells were injected subcutaneously in the right flank of NOD-SCID mice. On days 15, 20 and 25 the mice were injected (10mg/kg) intraperitoneously with: i) microRNA negative control (miR-NC); ii) antisense-microRNA negative control (as-miR NC); iii) miR-24 and/or miR-629; iv) as-miR-24 and/or as-miR-629; v) miR-24 and miR-629 (five mice/group). Tumor volume was monitored for 55 days.

Also, male mice on a C57BL/6 background were maintained in filter-topped cages and were fed standard rodent chow and water ad libitum. To induce hepatocellular carcinogenesis, mice were injected intraperitoneally (i.p.) with 25mg/kg of diethylnitrosamine (DEN) (Sigma) at 15 days of age. The mice were observed for development of tumors every 4 weeks and tumors were identified 32 weeks (~8 months) post DEN treatment. At weeks 1, 4, 8, 12, 24 and 32 mice (5 mice/group) were sacrificed and liver tissues were collected. Ricin A chain was used to eliminate Kuppfer cells and endothelial cells and purify hepatocytes (Johnston DE & Jasuja R. Hepatology,

20(2):436-44, 1994). HNF4α and IL6R mRNA expression levels and miR-124 and miR-24 levels were assessed by real-time PCR analysis in the purified hepatocytes.

Therapeutic protocol: 15 male C57BL/6 mice were DEN-treated and 32 weeks post treatment they were randomly distributed into 3 groups. The first group of mice did not receive any treatment, the second group of mice was treated with 10mg/kg microRNA-negative control (miR-NC) and the third group of mice was treated with 10mg/kg miR-124 (Exiqon Inc). MiR-NC and miR-124 were encapsulated in liposomes (Invivofectamine 2.0, Invitrogen) prior to injections. These liposomes have been designed for systemic microRNA delivery with high in vivo transfection efficiency in the liver following tail vein injection. Briefly, Invivofectamine 2.0 reagent is mixed with the microRNA and they are incubated for 30 min at 50^{o}C. MicroRNAs in a volume of 200ul were injected in the tail vein of the mice. MicroRNA treatments were performed in a weekly basis (week 33, 34, 35). At week 36, mice were sacrificed and tumor burden was assessed. Externally visible tumors larger than 0.5mm were counted using stereo microscopy. Primary hepatocytes were purified as described above and RNA and protein were extracted. MiR-124 levels, HNF4α and IL6R mRNA levels were assessed by real-time PCR analysis, while STAT3 and phosphorylated STAT3 (Tyr705) status were assessed by western blot analysis.

Prevention protocol: 15 male C57BL/6 mice were DEN-treated and 12 weeks post treatment they were randomly distributed into 3 groups. The first group of mice did not receive any treatment, the second group of mice was treated with 10mg/kg microRNA-negative control (miR-NC) and the third group of mice was treated with 10mg/kg miR-124. MicroRNAs were encapsulated in liposomes as described above and treatments were performed biweekly (weeks 12, 14, 16, 18, 20, 22, 24, 26, 28, 30). At week 32, mice were sacrificed and tumor burden was assessed as described above. Primary hepatocytes were purified from mouse liver tissues, RNA was extracted and miR-124 expression levels were assessed by real-time PCR analysis (Ambion Inc).

Hepatocyte Conditional knock-out mouse experiment. $STAT3^{f/f}$ and $STAT3^{\Delta hep}$ male mice were treated with 25 kg/mg DEN when 15 days old. Mice were sacrificed 8 months later and tumor tissues from DEN-treated $STAT3^{\Delta hep}$ mice and DEN-treated $STAT3^{f/f}$ mice were collected (same tumors as the ones described in figures 6B, C of He et al., Cancer Cell, 2010). From 4 tumors derived from the $STAT3^{\Delta hep}$ mice and 4 tumors from $STAT3^{f/f}$

mice, we extracted RNA and tested the expression levels of HNF4α, miR-124, miR-24 and miR-629. The mouse experiments have been performed in accordance with the University of California San Diego (UCSD) and Dana-Farber Cancer Institute Animal Care Committee guidelines.

Patient Samples

RNA samples: RNAs from 45 hepatocellular carcinomas and 12 normal tissues were purchased from Biochain (Hayward, CA) and Origene (Rockville, MD). The expression levels of miR-24, IL6R, miR-124 and HNF4α were analyzed by real-time RT-PCR in all the tissues described above. Each sample was run in triplicate and the data represent the mean ± SD.

Tissue samples: FFPE samples from 8 normal liver and 31 hepatocellular carcinomas were provided by Stanford University School of Medicine. An informed consent has been obtained from all subjects and the study has been approved by the Institutional Review Board of Stanford University. Hepatocytes were captured by laser capture microdissection and RNA (RNeasy FFPE Kit, cat no. 73504, Qiagen Inc) and protein (QProteome FFPE Tissue Kit, cat no. 37623, Qiagen Inc) were extracted. All the samples were negative for CD45 expression, validating the absence of immune cells in these samples. RNA was used to perform real-time PCR analysis for HNF4a, miR-124, IL6R, miR-24 and miR-629 and protein was used to perform ELISA analysis for IL6, IL6R and phosphorylated STAT3.

***In situ* microRNA hybridization**

Double-DIG labeled Mircury LNA probes for the detection of hsa-miR-24 (18121-15, Exiqon), hsa-miR-124 (88066-15, Exiqon) and hsa-miR-629 (38699-15, Exiqon) by *in situ* hybridization, were used as previously described (Iliopoulos et al., 2009b) with modifications. FFPE sections of normal liver and HCCs were deparaffinized with xylene (3x5 min), followed by treatment with serial dilutions of ethanol (3x100%, 2x96% and 3x70%) and by two changes of DEPC-PBS. Tissues were then digested with proteinase K (15 μg/ml) for 20 min at 37^{o}C, rinsed with 3xDEPC-PBS. Sections were dehydrated with 2x70%, 2x96% and 2x100% ethanol, air-dried and hybridized for 1 hour with the hsa-miR-24, hsa-miR-124 or hsa-miR-629 probe (40 nM) or the double-DIG labeled U6

Control Probe (1 nM) (99002-15, Exiqon) diluted in microRNA ISH buffer (90000, Exiqon), at 60, 60, 55 and 53^{o}C, respectively. Following hybridization, sections were rinsed twice with 5XSSC, 2x1XSSC and 3x0.2XSSC, 5 min each, at the hybridization temperatures and PBS. The slides were incubated with blocking solution (11585762001, Roche) for 15 min and then with anti-DIG antibody (1:800) in 2% sheep serum (013-000-121, Jackson Immunoresearch) blocking solution for 1 hour, at RT. Following three washes with PBS-T (PBS, 0.1% Tween-20), slides were incubated with the AP substrate buffer (NBT-BCIP tablet [11697471001, Roche] in 10 ml of 0.2 mM Levamisole [31742, Fluka]) for 2 hours at 30^{o}C in the dark. The reaction was stopped with 2 washes of AP stop solution (50 mM Tris-HCl, 150 mM NaCl, 10 mM KCl) and 2 washes with water. Tissues were counter stained with Nuclear Fast Red for 1 min and rinsed with water. Sections were dehydrated with 2x70%, 2x96% and 2x100% ethanol and mounted with coverslips in Eukitt mounting medium (361894G, VWR). Images were captured with a Nikon 80i Upright Microscope equipped with a Nikon Digital Sight DS-Fi1 color camera, using the NIS-Elements image acquisition software. All images were captured and processed using identical settings.

Immunohistochemistry

For tissue immunostaining for HNF4α and phospho-STAT3, FFPE sections of normal liver and HCCs were deparaffinized with xylene (3x5 min) followed by treatment with serial dilutions of ethanol (100%, 100%, 95% and 95%, 10 min each) and by two changes of ddH_2O. Antigen unmasking was achieved by boiling the slides (95-99^{o}C) for 10 min, in 10 mM sodium citrate (for HNF4α), pH 6.0, or 1 mM EDTA pH 8.0 (for phospho-STAT3). Sections were rinsed three times with ddH_2O, immersed in 3% H_2O_2 for 20 minutes, washed twice with ddH_2O and once with TBS-T (TBS, 0.1% Tween-20) and blocked for 1 hour with blocking solution (5% normal goat serum [5425] in TBS-T). HNF4A (3113, Cell Signaling Technology) and phospho-STAT3 (Tyr705) (9145, Cell Signaling Technology) antibodies were diluted 1:50 in Signal Stain antibody diluent (8112, Cell Signaling Technology) and incubated with the sections overnight at 4^{o}C. Following incubation with the antibodies, sections were washed three times, 5 min each, with TBS-T and incubated for 1 hour at room temperature with SignalStain Boost ([HRP, Rabbit] 8114, Cell Signaling Technology). Sections were washed three times, 5 min

each, with TBS-T, and stained with the DAB Peroxidase Substrate Kit (SK-4100, Vector Laboratories) for 30 min, washed and counterstained with the hematoxylin QS (H-3404, Vector Laboratories). Finally, tissues were dehydrated and mounted in Eukitt medium. Images were captured with a Nikon 80i Upright Microscope equipped with a Nikon Digital Sight DS-Fi1 color camera, using the NIS-Elements image acquisition software. All images were captured and processed using identical settings in the Nikon Imaging Center at Harvard Medical School.