Supporting Information

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SI Materials and Methods

Cell Lines and Transfections. The HB cell lines Huh6 and HepG2 were grown in RPMI medium supplemented with Glutamax (Invitrogen), 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. HepaRG cells were grown in William's medium supplemented with 10% (vol/vol) FBS, antibiotics, 5 μg/mL insulin, and 5×10^{-5} M hydrocortisone hemisuccinate as described (1). Cells were transfected with siRNA using InterferinTM (Ozyme) and harvested 24 h later for tumorigenic assays or 96–120 h later for RNA and protein analysis as described (2). Expression of miR-371, miR-372, and miR-373 was inhibited by transfection of 5–50 nM specific miRIDIAN micro-RNA Inhibitor (Dharmacon) with Lipofectamine 2000 (Invitrogen), and cells were harvested 24–48 h later for in vitro and in vivo assays.

miR Cluster Cloning and Cell Transduction. For simultaneous expression of the three miRs from the miR-371–3 cluster, which spans a large genomic locus, sequences of the mature form of each miR flanked by 200-bp genomic sequences were PCR-amplified and cloned into the pMSCV-PIG-IRES-GFP retroviral vector, respecting the order found on the chromosome. Primers are described below. All constructs were sequence-verified. Retroviral vectors were transfected in amphotropic packaging Phoenix A cells, and the supernatant was used to transduce hepatoma cells. At 48 h after infection, cells were selected by incubation with 4 μg/mL puromycin for 1 wk.

Quantitative ChIP. For ChIP analysis in hepatoma cells, we used 1×10^6 cells and 2 μg of Myc antibody (N-262; Santa Cruz) for each sample. For ChIP on HB specimens, tissue was minced in PBS and incubated 15 min in 1% formaldehyde with rotation at room temperature. Approximately 30 mg of tissue was disrupted, and nuclei were isolated by sequential use of A and B dounce homogenizers. From this step on, the ChIP protocol for cell lines was applied. In qPCR assays, 1 or 2 μL of ChIP or Input sample was used for each reaction in triplicate experiments using SybrGreen MasterMix (Applied Biosystems) with a temperature ranging from 60–64 °C and an ABI PRISM 7900HT S instrument. Mean values were determined from at least three independent ChIP experiments. Primers can be found below.

List of Primers Used for Quantitative ChIP and miR Cluster Cloning. Genomic enrichment was measured with the following primers (f: forward, r: reverse, ps: product size):

- miR-371–3 E-box 1: f: gtccactccttgcctcgata, r: gaattgcaggagagccagag, ps: 169 bp
- miR-371–3 E-box 2: f: gtccctcacacgtgttcctt, r: agcccttgatgagctgttga, ps: 177 bp
- miR-371–3 E-box 3: f: accacgcctggctaattttt, r: cagtgatagggcaaacagca, ps: 184 bp
- NUCL intron (E-box): f: gggtggagagatgagaccaa, r: actccgactagggccgatac, ps: 173 bp
- NUCL promoter (−400 bp): f: gagggcagagaaggagaggt, r: ccctgctggagagaaatctg, ps: 198 bp

Primers to amplify LIN28B are described by Chang et al. (3). The following primers were used to clone miR clusters:

- miR-371–3: f: gcctcgagcctcatggcttgcatctggagg, r: gcgaattcctgcaggtgaaccccgtatcct, ps: 1,168 bp
- mir100: f: gcctcgaggggacgaagtcctttccatt, r: gcgaattcaggtctccttcctccacctc, ps: 340 bp
- let-7a-2: f: gccaattgttgctcccttcatgttttca, r: gcgaattctaaaataccataaaataat, ps: 375 bp
- mir125b-1 f: gccaattgcggaatctcaattttgtgaagg, r: gcgaattcctgccactctctggtcacct, ps: 365 bp
- miR-100+let-7a-2+miR-125b-1: ps: 1,080 bp

Microarrays and Statistical Evaluation. miRs were profiled using miRNA microarray V 2.0, which carries 250 nonredundant human miRs, at the Microarray Shared Resource, Comprehensive Cancer Center, Ohio State University Medical Center. Raw data were normalized by the print-tip loess method (4). For supervised analysis, we compared miR expression between two classes of samples using the Student's *t* test with a random variance model option (BRB ArrayTools software, version 3.6.0a; http://linus.nci.nih.gov/BRB-ArrayTools.html). For tumor classification based on miR expression profiles, we used the Class prediction tool of BRB Array Tools, keeping the classification provided by the majority of algorithms.

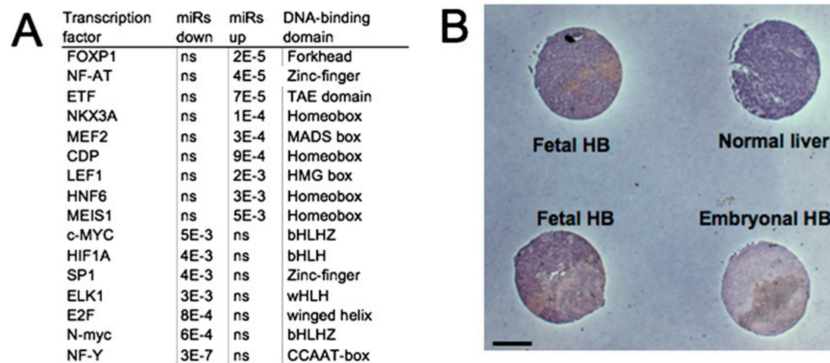
The HCC miR database has been previously published (5). Sample data and annotation can be found in the Gene Expression Omnibus database (accession no. GSE6857). Hierarchical clustering with the four-miR list was performed using dChip software (http://biosun1.harvard.edu/complab/dchip/). GO and KEGG pathway analysis was carried out as described (2). For GSEA analysis of Myc target miRs, the complete nonfiltered set of miRs was used as "gene set L," and miRs used as "gene set S" are shown in Fig. 2*B*.

Cross-Comparison of mRNA and miR Datasets. To obtain a database of predicted targets for each miR, we combined four sources: Pictar (http://pictar.mdc-berlin.de/), TargetScan 5.1 (http://www.targetscan.org/), mirbase miranda 3.0 (http://www.microrna.org/), and MirTarget 2 V3.0 (http://mirdb.org/). Using samples for which both gene and miR expression was available, we computed the Pearson coefficient of correlation between the intensity values of each gene probe set and each miR probe. For each miR probe corresponding to a mature sequence, we selected the anticorrelated gene expression probe sets showing a correlation score ≤0.45. This list was then filtered using its intersection with the database of predicted targets for the corresponding miR. Finally, for each miR, the final list of predicted targets with anticorrelated expression was used for enrichment analyses, utilizing pathways and gene sets from the KEGG (http://www.genome.jp/kegg/) and GO (http://www.geneontology.org) databases.

For transcription factor-binding predictive analysis, nonredundant databases for miRs up-regulated in C2-type HB by comparison with C1 were obtained by taking into account all predicted target genes that showed inversely correlated expression with the corresponding miR. The same strategy was applied to obtain consensus gene lists for down-regulated miRs. Transcription factor enrichment was measured using the EXPression ANalyzer and DisplayER (EXPANDER) gene expression analysis and visualization software (6).

1. Parent R, Marion MJ, Furio L, Trépo C, Petit MA (2004) Origin and characterization of a human bipotent liver progenitor cell line. *Gastroenterology* 126:1147–1156.
2. Cairo S, et al. (2008) Hepatic stem-like phenotype and interplay of Wnt/beta-catenin and Myc signaling in aggressive childhood liver cancer. *Cancer Cell* 14:471–484.
3. Chang TC, et al. (2009) Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. *Proc Natl Acad Sci USA* 106:3384–3389.
4. Hua YJ, Tu K, Tang ZY, Li YX, Xiao HS (2008) Comparison of normalization methods with microRNA microarray. *Genomics* 92:122–128.

5. Budhu A, et al. (2008) Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology* 47:897–907.
6. Shamir R, et al. (2005) EXPANDER—An integrative program suite for microarray data analysis. *BMC Bioinformatics* 6:232–243.



Transcription factor	miRs down	miRs up	DNA-binding domain
FOXP1	ns	2E-5	Forkhead
NF-AT	ns	4E-5	Zinc-finger
ETF	ns	7E-5	TAE domain
NKX3A	ns	1E-4	Homeobox
MEF2	ns	3E-4	MADS box
CDP	ns	9E-4	Homeobox
LEF1	ns	2E-3	HMG box
HNF6	ns	3E-3	Homeobox
MEIS1	ns	5E-3	Homeobox
c-MYC	5E-3	ns	bHLHZ
HIF1A	4E-3	ns	bHLH
SP1	4E-3	ns	Zinc-finger
ELK1	3E-3	ns	wHLH
E2F	8E-4	ns	winged helix
N-myc	6E-4	ns	bHLHZ
NF-Y	3E-7	ns	CCAAT-box

Fig. S1. (*A*) Representative transcription factors significantly associated with putative miR target genes showing negatively correlated expression with cognate miR. *P* values are shown for correlations with miRs up-regulated (miRs up) or down-regulated (miRs down) in the C2-type HB subtype compared with the C1 subtype. ns, not significant. (*B*) mir-122 expression in normal liver and HB measured by ISH. (Scale bar, 3 mm.)

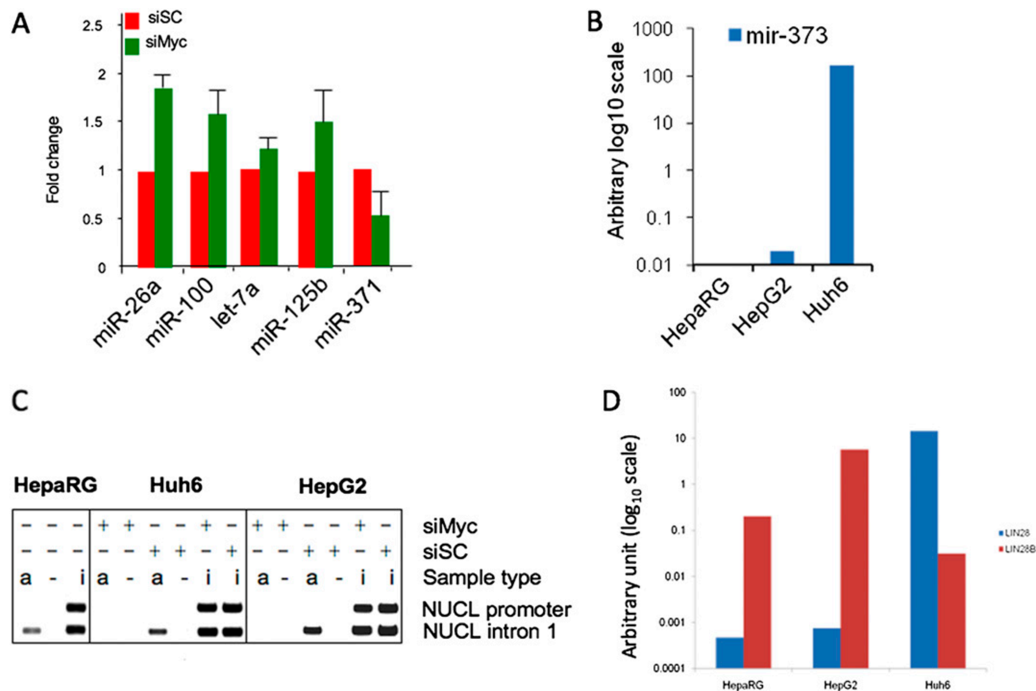


Fig. S2. (*A*) siRNA-mediated inhibition of Myc in HepG2 cells reduces expression of miR-371 but activates expression of miR-26a, miR-100, let-7a, and miR-125b. qPCR assays after 96 h of treatment with Myc targeting or scrambled siRNA are shown as the mean ± SD of three independent experiments. (*B*) Expression of miR-371 analyzed by qPCR in HepaRG, HepG2, and Huh6 cell lines. (*C*) ChIP analysis of Myc binding to NUCL gene promoter and intron 1. a, +Myc antibody; –, no antibody; i, input DNA. (*D*) Analysis of LIN28 and LIN28B expression in hepatoma cell lines by qPCR.

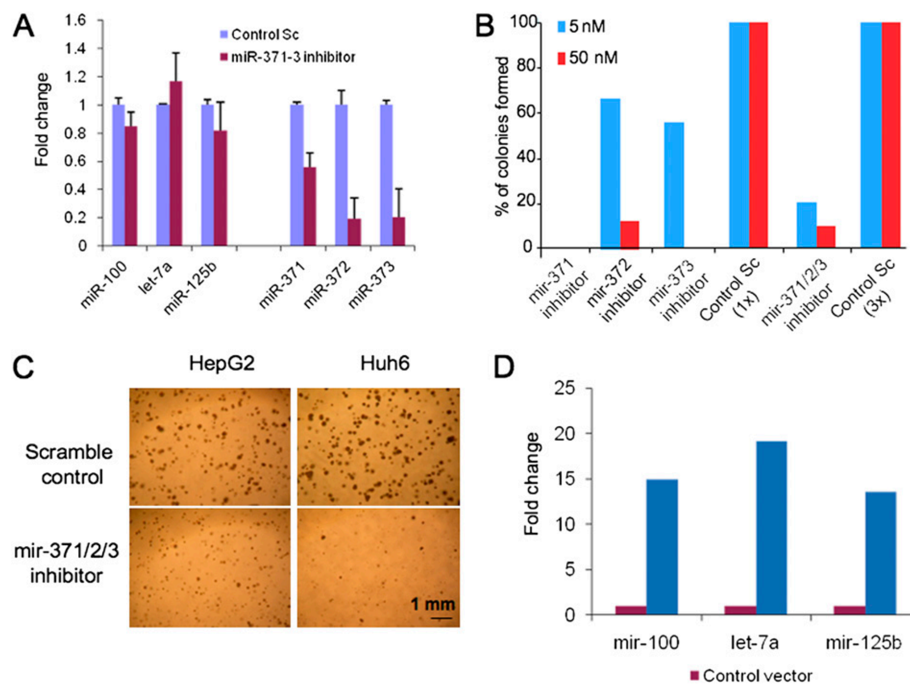


Fig. S3. (*A*) qPCR analysis of miR expression in Huh6 cells on inhibition of miR-371–3. Results from three independent experiments (mean ± SD) were normalized against miR expression in cells transfected with scrambled control. (*B*) Representative soft agar assay in Huh6 cells treated with different concentrations of miR-371, miR-372, and miR-373 inhibitor administered separately or together. (*C*) Soft agar assay on HepG2 and Huh6 cells using mir-371/2/3 inhibitors or scramble negative control oligonucleotides. These results refer to a 3-wk incubation after transfection with a 10-nM final concentration of each miR inhibitor or with a final 30-nM scramble control. (*D*) Ectopic miR expression in Huh6 cells infected with a retroviral vector expressing miR-100, let-7a-2, and miR-125b-1.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S3 (DOC)
Table S4 (DOC)
Table S5 (DOC)
Table S6 (DOC)