

An HNF4 α -miRNA Inflammatory Feedback Circuit Regulates Hepatocellular Oncogenesis

Maria Hatzia Apostolou,^{1,2} Christos Polyta rchou,^{1,2} Eleni Aggelidou,⁴ Alexandra Drakaki,⁵ George A. Poultsides,⁶ Savina A. Jaeger,⁷ Hisanobu Ogata,⁸ Michael Karin,⁸ Kevin Struhl,³ Margarita Hadzopoulou-Cladaras,^{4,9} and Dimitrios Iliopoulos^{1,2,9,*}

¹Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute

²Department of Microbiology and Immunobiology

³Department of Biological Chemistry and Molecular Pharmacology

Harvard Medical School, Boston, MA 02115, USA

⁴Department of Genetics, Developmental and Molecular Biology, Laboratory of Developmental Biology, School of Biology, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece

⁵Division of Hematology/Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA 02215, USA

⁶Department of Surgery, Stanford University Medical Center, Stanford, CA 94305, USA

⁷Department of Developmental Molecular Pathways, Novartis Institute for Biomedical Research, Cambridge, MA 02139, USA

⁸Laboratory of Gene Regulation and Signal Transduction, School of Medicine, University of California, San Diego, La Jolla, CA 92093, USA

⁹These authors contributed equally to this work

*Correspondence: dimitrios_iliopoulos@dfci.harvard.edu

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SUMMARY

Hepatocyte nuclear factor 4 α (HNF4 α) is essential for liver development and hepatocyte function. Here, we show that transient inhibition of HNF4 α initiates hepatocellular transformation through a microRNA-inflammatory feedback loop circuit consisting of miR-124, IL6R, STAT3, miR-24, and miR-629. Moreover, we show that, once this circuit is activated, it maintains suppression of HNF4 α and sustains oncogenesis. Systemic administration of miR-124, which modulates inflammatory signaling, prevents and suppresses hepatocellular carcinogenesis by inducing tumor-specific apoptosis without toxic side effects. As we also show that this HNF4 α circuit is perturbed in human hepatocellular carcinomas, our data raise the possibility that manipulation of this microRNA feedback-inflammatory loop has therapeutic potential for treating liver cancer.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the main type of liver cancer and the third most common cause of cancer mortality worldwide. The major risk factor for HCC is chronic hepatitis, due to hepatotropic viruses (HBV, HCV) (El-Serag and Rudolph, 2007), but the molecular mechanisms leading to HCC have not been well characterized. Hepatocellular carcinogenesis involves many genetic and epigenetic alterations and is influenced by environmental factors. Genes such as *c-myc*, cyclin D1, p53, p16, E-cadherin, and PTEN have been linked to hepatocarcino-

genesis (Villanueva et al., 2007). Persistent inflammation also impacts the course of liver tumor development (Coussens and Werb, 2002), and chronic inflammatory stimuli and increased STAT3 activation recapitulate hepatic oncogenesis in various animal models (He et al., 2010). In addition, the inflammatory responses induced by obesity or administration of the diethylnitrosamine (DEN) are known to promote HCC in mice (Park et al., 2010; Maeda et al., 2005).

HNF4 α is a member of the nuclear receptor superfamily of ligand-dependent transcription factors (NR2A1) that is enriched in liver tissue (Zhong et al., 1993). HNF4 α is indispensable for development and maintenance of the hepatic epithelium (Parviz et al., 2003) and also has links to a variety of human diseases, including diabetes, colitis, and cancer. A number of mutations within the HNF4A gene are considered to contribute to several forms of maturity-onset diabetes in children (Gupta and Kaestner, 2004). Suggesting a potential link between HNF4 α and inflammation, genome-wide association studies have identified HNF4A as a susceptibility locus for ulcerative colitis (Barrett et al., 2009), and recent evidence supports an oncogenic role for HNF4 α in intestinal cancer (Darsigny et al., 2010). But conflicting reports have assigned HNF4 α both tumor-promoting and tumor-suppressing roles in liver cancer (Xu et al., 2001; Yin et al., 2008).

Here, we show that HNF4 α is a key regulator of hepatocellular carcinogenesis. During hepatocellular transformation, transient inhibition of HNF4 α becomes a stable event, with a feedback loop consisting of miR-124, IL6R, STAT3, miR-24, and miR-629 maintaining the hepatocyte-transformed phenotype in vitro and in vivo. Perturbation of this network, through miR-124 systemic administration, prevents and suppresses HCC development in a murine liver cancer model. Components of the HNF4 α feedback loop circuit are differentially expressed in

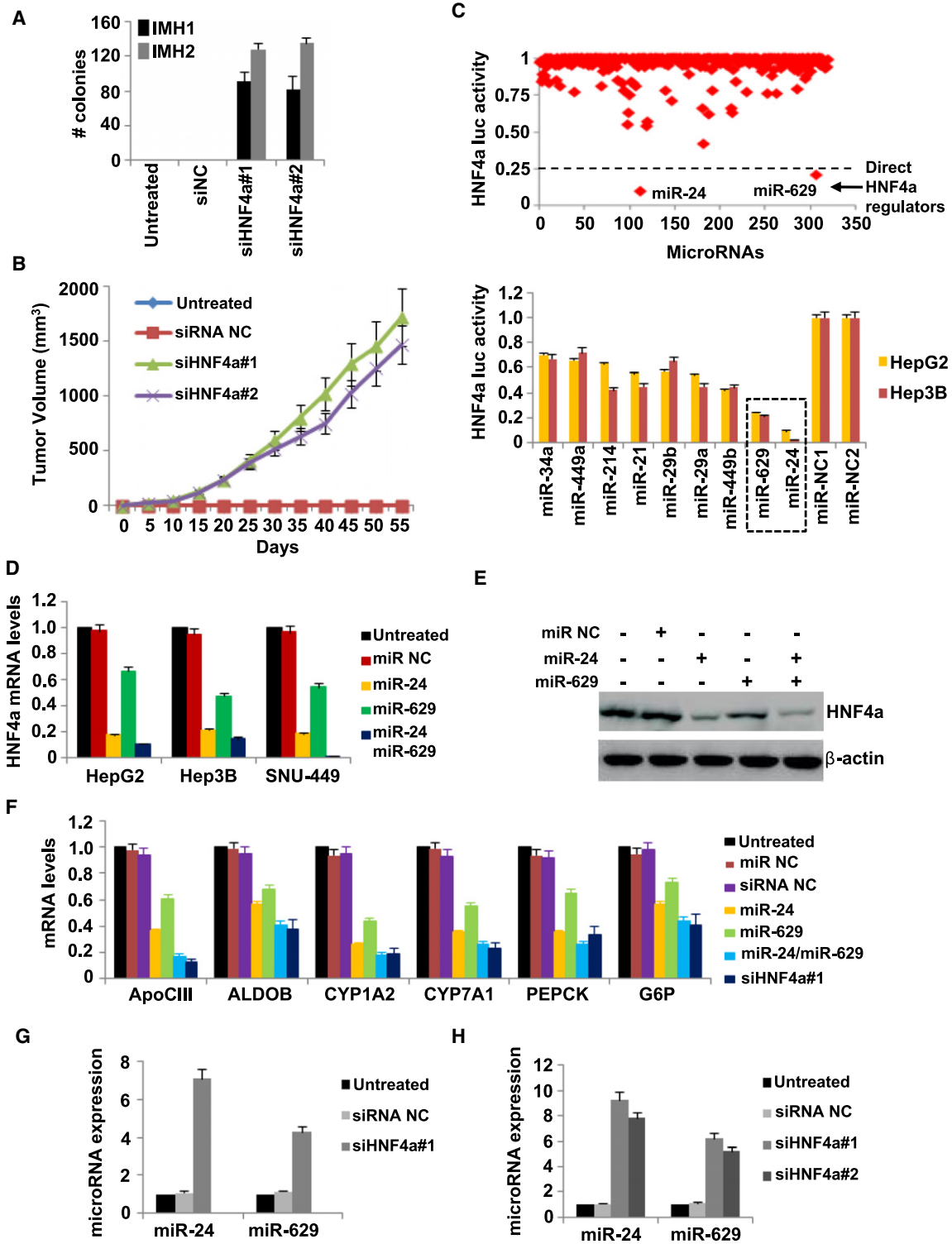


Figure 1. HNF4 α Suppression through miR-24 and miR-629 Induces Hepatocellular Transformation

(A) Soft agar colony assay of nontransformed immortalized hepatocytes (IMH1, IMH2) treated for 48 hr with siRNA-negative control (siNC) or two different siRNAs against HNF4 α (siHNF4 α #1, siHNF4 α #2). Colonies (mean \pm SD) 50 μ m were counted using a microscope 20 days later.

(B) Tumor volume (mean \pm SD) in mice injected with IMH1 cells untreated or treated for 48 hr with siRNA NC, siHNF4 α #1, or siHNF4 α #2.

(C) Effects of microRNAs (primary screen) on HNF4 α luciferase activity in HepG2 cells (top). The top nine hits identified from the primary microRNA library screen were tested in secondary screen in HepG2 and Hep3B cells (bottom).

human hepatocellular carcinomas relative to normal liver tissues. Though the epigenetic switch described here resembles an epigenetic switch that converts a nontransformed breast cell line into a stably transformed line that relies on an inflammatory feedback loop involving STAT3 (Iliopoulos et al., 2009, 2010), the microRNA, transcription factors, and target genes mediating these epigenetic switches differ considerably in the breast and liver contexts. Overall, our data suggest that epigenetic switches are regulatory events that are essential for cancer initiation and maintenance in addition to mutational events.

RESULTS

Transient Inhibition of HNF4 α Induces Hepatocellular Oncogenesis

To elucidate the role and function of HNF4 α in liver cancer initiation, we modulated its expression in nontransformed immortalized human hepatocytes (IMH). We found that HNF4 α inhibition transformed IMH cells and increased their invasiveness (Figures 1A and 1B and Figures S1A–S1C available online). Strikingly, transient inhibition of HNF4 α was sufficient to induce transformation of IMH cells and promote tumor formation in immunodeficient mice (Figures 1A and 1B). In these tumors (day 55), HNF4 α mRNA expression was still suppressed (Figure S1D), suggesting that inhibition of HNF4 α initiates a feedback loop that continuously suppresses HNF4 α expression and induces a stable transformed phenotype. In accordance with the data from our primary IMH cells, transient inhibition of HNF4 α increased colony formation and invasiveness of HepG2 and SNU-449 cancer cells (Figures S1E and S1F) and decreased expression levels of HNF4 α direct metabolic target genes (Figure S1G). Overall, these data suggest that HNF4 α inhibition induces transformation of immortalized hepatocytes through a feedback regulatory mechanism.

miR-24 and miR-629 Suppress Directly HNF4 α Expression during Hepatocellular Transformation

How is HNF4 α suppression triggered and maintained during hepatocellular transformation? Recently, we and others have described the existence of dynamic microRNA-transcription factor networks in a variety of cancers (Iliopoulos et al., 2009, 2010; Kent et al., 2010). To identify microRNAs that regulate directly HNF4 α expression, we performed a microRNA-based genetic screen (Figure 1C, top). MicroRNAs that inhibited HNF4 α 3'UTR luciferase activity by more than 75% were scored as positive hits. These were further validated in HepG2 and Hep3B cells, seeded in 6-well plates, according to the same criteria (Figure 1C, bottom). Our approach resulted in the identi-

fication of two microRNAs, miR-24 and miR-629, as direct regulators of HNF4 α expression.

Several lines of evidence indicate that miR-24 and miR-629 target HNF4 α directly, binding to its 3'UTR. Sequence complementarity analysis revealed that HNF4 α is a gene target of miR-24 and miR-629, and upon overexpression of miR-24 or miR-629, HNF4 α mRNA levels are reduced 5-fold and 2-fold, respectively (Figure 1D). In addition, HNF4 α protein levels drop (Figure 1E), and the direct downstream targets are downregulated by miR-24 and miR-629 (Figure 1F). In addition, combined expression of these two miRNAs resembles the effects of HNF4 α knockdown (Figure 1F).

Transient inhibition of HNF4 α by siRNA resulted in upregulation of both miR-24 and miR-629 in IMH cells (Figure 1G). We also identified increased expression of miR-24 and miR-629 in tumors derived from IMH cells treated with two different siRNAs against HNF4 α (Figure 1H). Taken together, these data suggest that both microRNAs regulate directly HNF4 α expression and are part of the feedback loop circuit.

miR-24 and miR-629 Play a Key Role in Hepatocellular Cancer Initiation and Growth

To assess the functional role of miR-24 and miR-629 in tumorigenicity, we tested whether their overexpression can transform two distinct immortalized hepatocyte cell lines. Expression of miR-24 and/or miR-629 is sufficient for hepatocellular transformation and colony formation in soft agar (Figure 2A). Though miR-24 has a stronger effect than miR-629, the combination of the two microRNAs closely resembles HNF4 α knockdown. The ability of miR-24 or miR-629 to induce transformation in vitro led us to extend our results and examine their ability to regulate tumor initiation in vivo. Overexpression of miR-24 or miR-629, to a lesser extent, was sufficient for the induction of tumor initiation and growth (Figure 2B). These observations indicate that transient expression of either miR-24 or miR-629 is sufficient to induce stable transformation of hepatocytes in vitro and in vivo. Reduced HNF4 α expression in miR-24/miR-629-treated tumors (Figure 2C) also indicates that both microRNAs cooperatively suppress HNF4 α expression inducing a stable transformed state.

To address the functional role of miR-24 and miR-629 in the maintenance of the transformed phenotype, we tested the effects of their upregulation on the tumorigenicity of hepatocellular cancer cells. Overexpression of miR-24 or miR-629 in HepG2 and SNU-449 cells increased their ability to form colonies (Figure 2D) and their invasive capacity (Figure 2E). As expected, a combination of the two microRNAs exhibited the same effects with HNF4 α inhibition. To delineate the role of miR-24 and miR-629 in HCC growth in vivo, we performed xenograft experiments

(D) HNF4 α mRNA levels (mean \pm SD of three independent experiments) assessed by real-time RT-PCR analysis in HepG2, Hep3B, and SNU-449 cells untreated or treated with 100 nM miR NC or miR-24 and/or miR-629 for 48 hr.

(E) HNF4 α protein levels in HepG2 cells untreated or treated with 100 nM miR NC or miR-24 and/or miR-629 for 48 hr.

(F) mRNA levels of HNF4 α direct targets (mean \pm SD of three independent experiments) assessed by real-time RT-PCR analysis in HepG2 cells untreated or treated with 100 nM miR NC or miR-24 and/or miR-629 or siRNA NC or siRNA or siHNF4 α #1 for 48 hr.

(G) miR-24 and miR-629 expression levels (mean \pm SD of three independent experiments) assessed by real-time RT-PCR analysis in IMH1 cells that were untreated or treated for 48 hr with siRNA NC or siHNF4 α #1.

(H) miR-24 and miR-629 expression levels (mean \pm SD of three independent experiments) assessed by real-time RT-PCR analysis in tumors derived from injected IMH1 cells that were untreated or treated for 48 hr with siRNA NC or siHNF4 α #1 or siHNF4 α #2.

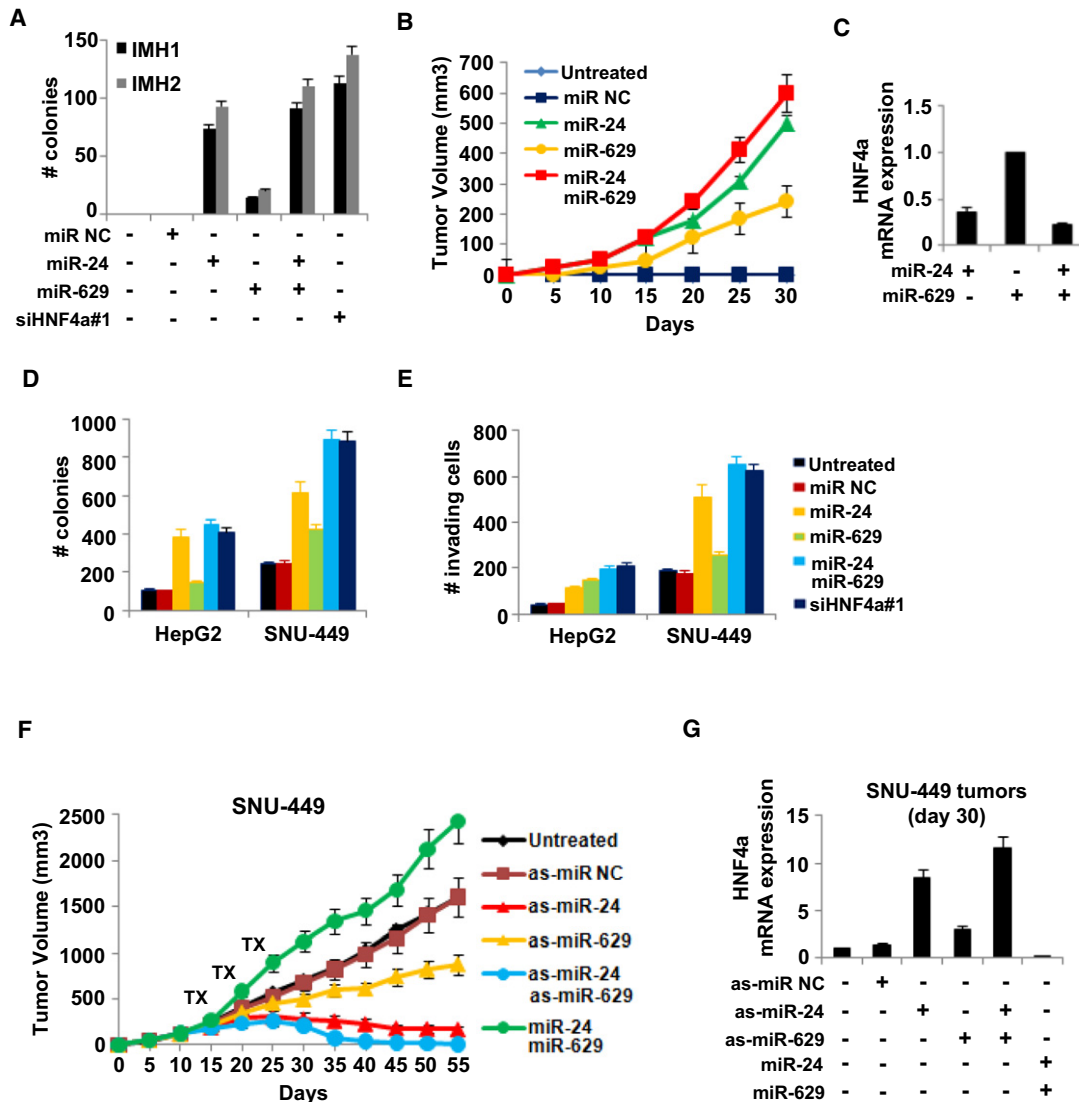


Figure 2. miR-24 and miR-629 Regulate the Induction and Stability of the Hepatocellular Transformed Phenotype

(A) Number of colonies (>50 μ m) (mean \pm SD) of IMH1 and IMH2 cells treated with 100 nM miR NC, miR-24, and/or miR-629 or siHNF4 α #1 for 48 hr. (B) Tumor volume (mean \pm SD) in mice injected with IMH1 cells untreated or treated for 48 hr with 100 nM miR NC or miR-24 and/or miR-629. (C) HNF4 α mRNA levels assessed by real-time RT-PCR analysis in tumors (day 30) derived from IMH1 cells untreated or treated for 48 hr with 100 nM miR-24 and/or miR-629. (D) Soft agar colony assay (mean \pm SD) and (E) invasion assay (mean \pm SD) of HepG2 and SNU-449 cells treated with 100 nM miR NC, miR-24, miR-629, or siHNF4 α #1 for 24 hr. (F) Tumor volume (mean \pm SD) in mice injected with SNU-449 cells and treated with as-miR NC or as-miR-24 and/or as-miR-629, or miR-24 and miR-629. (G) HNF4 α mRNA levels (mean \pm SD) in tumors (day 30) derived from mice treated with as-miR NC or as-miR-24 and/or as-miR-629 or miR-24 and miR-629.

in which SNU-449 cells were injected subcutaneously in immunodeficient mice (Figure 2F). We found that overexpression of miR-24 and miR-629 increased the growth of SNU-449 xenograft tumors (Figure 2F), whereas simultaneous inhibition of both microRNAs completely suppressed tumor growth. Are the effects of miR-24 and miR-629 on tumor growth related to HNF4 α expression? We tested HNF4 α mRNA levels in xenograft tumors (day 30) from the same mice, as described above. Tumors treated with the antisense microRNAs are smaller,

contain many apoptotic cells (Figure S2), and exhibit elevated HNF4 α mRNA levels (Figure 2G).

STAT3 Is a Direct Regulator of miR-24 and miR-629 Expression

According to our data, both miR-24 and miR-629 directly suppress HNF4 α expression, and they are activated by inhibition of HNF4 α expression in hepatocytes as part of the feedback loop circuit. We found that miR-24 and miR-629 are coordinately

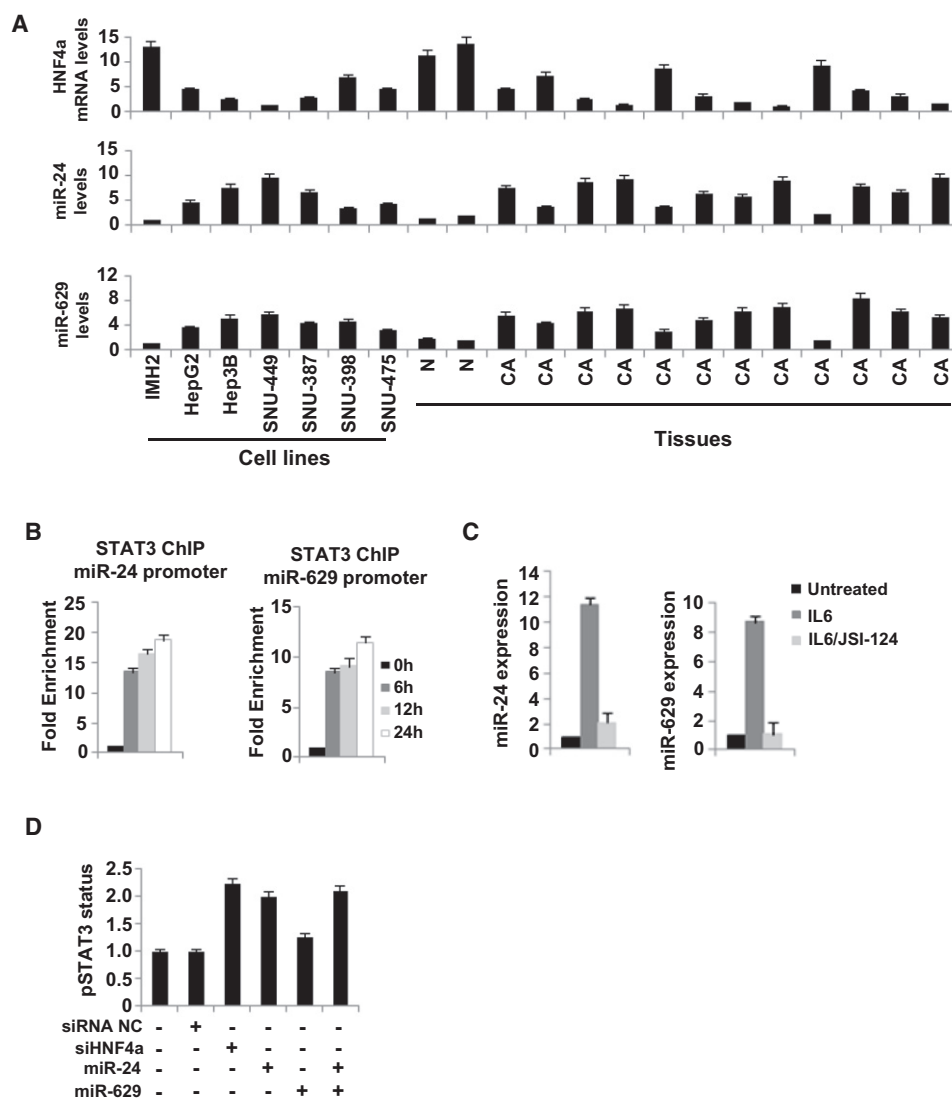


Figure 3. STAT3 Regulates miR-24 and miR-629 during Hepatocellular Transformation

(A) HNF4 α , miR-24, and miR-629 levels (mean \pm SD) in nontransformed immortalized hepatocytes (IMH2), different HCC lines, two normal liver tissues (N), and 12 hepatocellular cancer tissues (CA).

(B) STAT3 occupancy (fold enrichment) at the miR-24 and miR-629 loci, as determined by chromatin immunoprecipitation of crosslinked SNU-449 cells treated with IL6 (20 ng/ml) for 6, 12, or 24 hr.

(C) miR-24 and miR-629 expression levels (mean \pm SD) in SNU-449 cells treated with IL6 (10 ng/ml) for 24 hr or JSI-124 (5 μ g/ml) for 24 hr and then IL6 for 24 hr.

(D) STAT3 phosphorylation status (Tyr 705) assessed by ELISA in SNU-449 cells treated with 1 nM siRNA NC, siHNF4 α #1, miR-24, and/or miR-629 for 24 hr. The data are presented as mean \pm SD of three independent experiments.

upregulated in both hepatocellular cell lines and human tumors (Figure 3A). Examination of potential common transcription factor binding sites in miR-24 and miR-629 promoter areas revealed a highly conserved STAT3 binding motif in miR-24 promoter and a moderately conserved STAT3 motif in miR-629 promoter (Table S1). Chromatin immunoprecipitation (ChIP) analysis in SNU-449 cells revealed that, upon IL6 stimulation, STAT3 binds in miR-24 and miR-629 promoter regions, with binding to the highly conserved miR-24 site being stronger (Figure 3B). STAT3 activation by IL6 treatment resulted in upregulation of both miR-24 and miR-629 levels, whereas pharmaco-

logical inhibition of STAT3 (JSI-124) strongly reduced miR-24 and miR-629 expression levels (Figure 3C).

To determine whether STAT3 is a member of the HNF4 α feedback loop circuit, we measured STAT3 phosphorylation levels upon overexpression of miR-24 and/or miR-629 or inhibition of HNF4 α in SNU-449 cells (Figure 3D). Strikingly, all treatments significantly induced STAT3 phosphorylation when compared to the negative control samples. In accordance with our data above, miR-24 had a more pronounced effect (compared to miR-629), similar to that of HNF4 α knockdown and the combinatorial expression of the two microRNAs. These results strongly

suggest that these microRNAs, STAT3, and HNF4 α are part of an inflammatory feedback loop and not simply downstream effectors of IL6.

miR-124 Is a Direct Downstream Effector of HNF4 α Activity and Part of the Feedback Loop Network

Recent studies have identified microRNA-transcription factor feedback loops in cancer cells (Fabbri et al., 2011; Iliopoulos et al., 2010). To further unravel the mechanism by which inhibition of HNF4 α expression induces hepatocellular transformation through a feedback loop, we looked for HNF4 α -binding sites in miRNA promoters. Lever algorithm analysis revealed HNF4 α -binding sites in eight microRNA promoter areas (Table S2). ChIP analysis showed that HNF4 α binds strongly (15- to 25-fold enrichment) to miR-124 promoter in HepG2 and SNU-449 cells (Figure 4A), and inhibition of HNF4 α expression resulted in significant reduction of miR-124 levels (~5-fold) (Figure 4B). Similarly, miR-124 expression is significantly inhibited upon the combined overexpression of miR-24 and miR-629, and this inhibition is comparable with the one caused by HNF4 α knockdown. Based on our observation of an inverse correlation between STAT3 activation and HNF4 α expression, we examined how IL6 treatment influenced activity of a luciferase reporter construct containing the miR-124 promoter (Figure 4C). Treatment of HepG2 cells with IL6 significantly inhibited the activity of the miR-124 luciferase reporter, whereas there was no effect when the HNF4 α site was mutated.

As HNF4 α directly regulates miR-124 expression in HCC lines, we tested the possibility that miR-124 may mediate the HNF4 α -regulated inhibition of STAT3. Interestingly, STAT3 activation was induced upon miR-124 suppression when compared to the respective negative controls (Figure 4D). The above experiments suggest that miR-124 participates also in the HNF4 α feedback loop. To further show that miR-124 is a member of this loop, we examined IMH1 transformation efficiency upon inhibition of miR-124 expression. As expected, inhibition of miR-124 expression strongly induces colony formation, and this effect is reversed by STAT3 knockdown or combined suppression of miR-24 expression (Figure 4E). Likewise, suppression of miR-124 or knockdown of HNF4 α induces colony formation and invasiveness of HepG2 and SNU-449 cells, whereas overexpression of miR-124 in these cell lines reverses the phenotype (Figure S3). Taken together, these observations are consistent with a pathway in which STAT3 activation inhibits HNF4 α expression, which leads to suppressed expression of miR-124 and establishes an inflammatory feedback loop that is necessary and sufficient for human hepatocyte transformation.

miR-124 Targets IL6R and Consequently Modulates IL6R/STAT3 Pathway during Hepatocellular Transformation

Because STAT3 activation is suppressed by miR-124, we hypothesized that miR-124 might target one of the components of the IL6-STAT3 pathway. In support of this hypothesis, sequence complementarity and conservation analysis revealed that interleukin 6 receptor (IL6R) is a potential direct gene target of miR-124. Furthermore, miR-124 and IL6R expression levels

are inversely correlated in IMH1 cells and five hepatocellular cancer cell lines (Figure 4F). In addition, suppression of miR-124 expression, either directly by antisense miR-124 or indirectly by knockdown of HNF4 α , leads to induced expression of IL6R (Figure 4G). Conversely, overexpression of miR-124 significantly reduced IL6R mRNA and protein levels (Figures 4G and 4H). Also, miR-124 overexpression inhibits the activity of a luciferase reporter construct containing the IL6R 3'UTR and vice versa (Figure 4I). Next, phosphorylation of STAT3, a downstream target of IL6R, is induced by inhibition of miR-124 expression or knockdown of HNF4 α (Figure 4J). In addition to IL6R, we found that inhibition of miR-124 expression results in increased IL6 production (Figure S4A), suggesting that miR-124 regulates STAT3 activity by affecting the IL6-IL6R levels and pathway. Similar effects were identified when HNF4 α was suppressed. Specifically, HNF4 α inhibition resulted in increased levels of soluble IL6 and IL6R (Figures S4B and S4C), which, in turn, increased liver tumorigenicity (Figure S4D). These experiments support a central role for HNF4 α in regulating the IL6-STAT3 inflammatory response.

The Feedback Loop Involving HNF4 α , miR-124, IL6R, STAT3, miR-24, and miR-629 Is Required for the Induction and Maintenance of the Transformed Phenotype in Hepatocytes

To examine the dynamics of this circuit during the transformation of hepatocytes, IMH1 cells were transiently transfected with the respective microRNAs or siRNAs and 96–480 hr posttransfection were plated in soft agar and injected in mice (Figures S5A and S5B). Suppression of miR-124 or HNF4 α or overexpression of miR-24 or miR-629 induced hepatocellular transformation. We also find that the kinetics of STAT3 activation along with expression levels of miR-124, miR-24, miR-629, and HNF4 α demonstrate the establishment and maintenance of the regulatory loop even 480 hr after transfection (Figures S5C–S5G). In addition to transcriptional activation, we show that suppression of HNF4 α led to increased soluble IL6 and IL6R levels (Figure S5H), hepatocyte hyperproliferation, and decreased apoptosis (Figure S6). On the other hand, breaking the regulatory circuit by manipulation of different members of the loop blocked the stable transformed phenotype of human hepatocytes (Figures S5I and S5J). Overall, these data indicate that HNF4 α is a central regulator of hepatocyte growth and transformation.

HNF4 α -miRNA Inflammatory Circuit Is Perturbed during HCC Development in Mice

Building on our *in vitro* findings, we asked whether the HNF4 α circuit is perturbed during development of chemical-induced hepatocellular carcinogenesis *in vivo*. To exclude the possibility that the IL6/STAT3 pathway is activated by Kupffer cells, we examined the expression levels of HNF4 α , miR-124, IL6R, and miR-24 in purified hepatocytes derived from DEN-treated mice (Figure 5A). In accordance with our *in vitro* data, we identified that the HNF4 α -miRNA circuit is perturbed in hepatocytes during HCC development in mice. Interestingly, HNF4 α suppression started on week 4, whereas miR-24 was upregulated on week 24, when the tumors have already been formed. These data are consistent with the idea that early suppression of HNF4 α

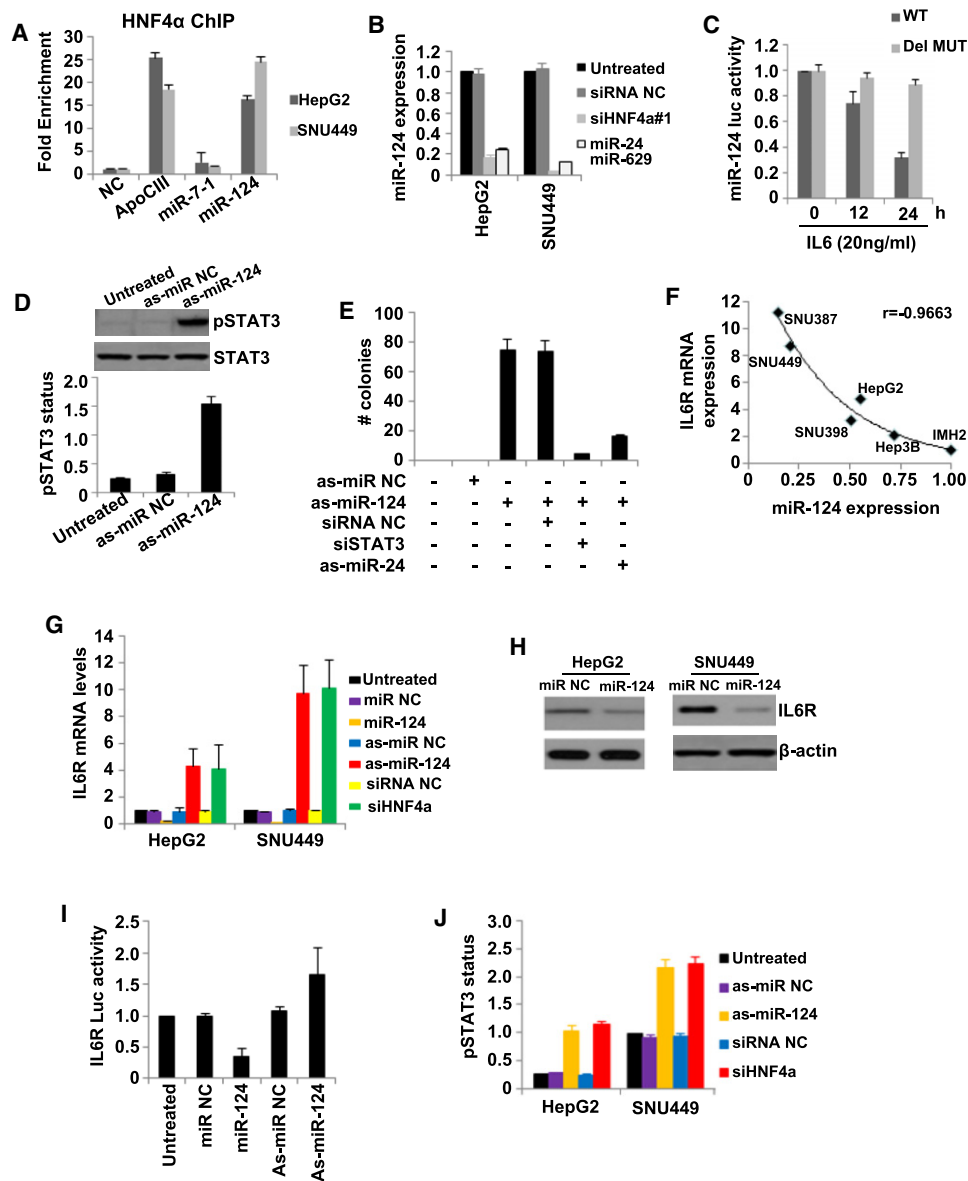


Figure 4. HNF4 α Binds and Regulates miR-124, which Controls Directly IL6R Expression in Hepatocytes

(A) HNF4 α occupancy (fold enrichment) (mean \pm SD) in ApoCIII, miR-7-1, and miR-124 promoter areas.

(B) miR-124 levels (mean \pm SD) in HepG2 and SNU-449 treated with siRNA NC, siHNF4 α #1, miR-24, and miR-629 for 24 hr.

(C) Luciferase activity (mean \pm SD) of a reporter construct harboring miR-124 promoter (wild-type or deletion mutant in the HNF4 α -binding site) 12 and 24 hr posttreatment with IL6 (10 ng/ml) in HepG2 cells.

(D) STAT3 phosphorylation status (Tyr 705) (mean \pm SD) evaluated by ELISA and western blot analyses after treatment with as-miR-NC or as-miR-124 for 24 hr in HepG2 cells.

(E) Number of colonies (mean \pm SD) of nontransformed immortalized hepatocytes (IMH1) treated with as-miR NC or as-miR-124 together with siRNA NC or siRNA against STAT3 (siSTAT3) or as-miR-24 for 24 hr. The data are presented as mean \pm SD of three independent experiments.

(F) miR-124 and IL6R levels in the indicated cell lines and a correlation coefficient (r) are shown.

(G) IL6R mRNA levels (mean \pm SD) in HepG2 and SNU-449 cells treated for 24 hr with miR-NC, miR-124, as-miR NC, as-miR-124, siRNA NC, and siHNF4 α .

(H) IL6R protein levels in HepG2 and SNU-449 cells treated for 24 hr with miR-NC or miR-124.

(I) Luciferase assay using a reporter construct containing the 3'UTR of IL6R, 24 hr after transfection with miR-NC, miR-124, as-miR NC, and as-miR-124. The data are presented as (mean \pm SD).

(J) STAT3 phosphorylation status (Tyr 705) evaluated by ELISA in HepG2 and SNU-449 cells treated for 24 hr with as-miR NC, as-miR-124, siRNA NC, and siHNF4 α . The data are presented as mean \pm SD of three independent experiments.

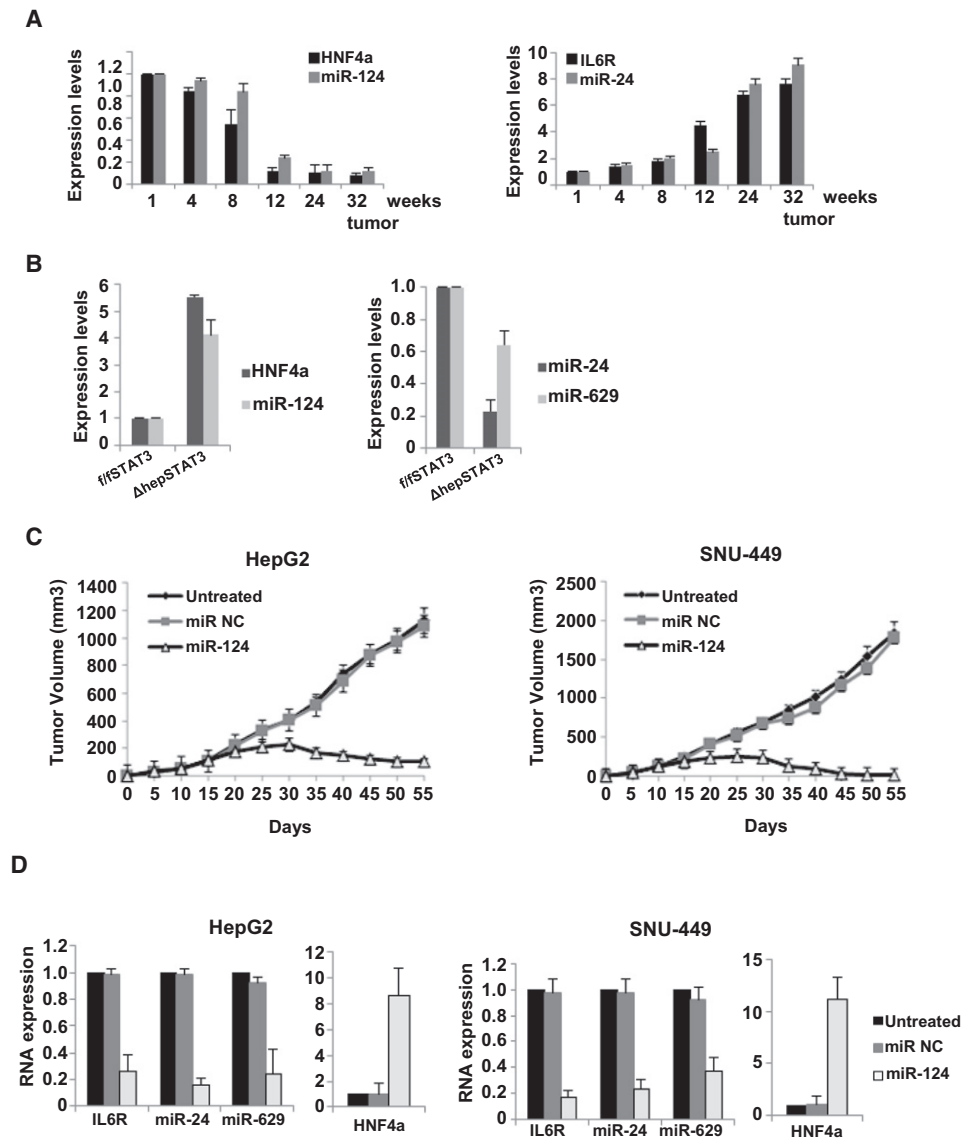


Figure 5. The HNF4 α Circuit Is Perturbed during HCC Development

(A) Assessment of HNF4 α , miR-124, IL6R, and miR-24 levels (mean \pm SD) in purified hepatocytes during DEN-induced liver carcinogenesis in mice. (B) Evaluation of HNF4 α mRNA levels and miR-124, miR-24, and miR-629 levels derived from DEN-treated male STAT3^{f/f} and STAT3^{Δhep} mice. The experiments have been performed in triplicate, and data show mean \pm SD. (C) Tumor volume (mean \pm SD) in mice injected with HepG2 and SNU-449 cells treated with miR NC or miR-124. Treatments were repeated every 5 days, and tumor volume was monitored every 5 days for 55 days. (D) IL6R, miR-24, miR-629, and HNF4 α levels (mean \pm SD) assessed by real-time RT-PCR analysis, in tumors (day 30) derived from mice treated with miR NC or miR-124.

leads to activation of the miRNA inflammatory circuit during HCC development.

In addition, we tested whether the HNF4 α -miRNA circuit is perturbed in hepatocyte-specific STAT3-deficient mice (STAT3^{f/f}/Alb-Cre = STAT3^{Δhep}). It is known that the DEN-treated STAT3^{Δhep} mice develop fewer and much smaller tumors in comparison to the DEN-treated STAT3^{f/f} mice (He et al., 2010). Consistent with our hypothesis, we identified that tumors derived from DEN-treated STAT3^{Δhep} mice had increased HNF4 α and miR-124 levels and decreased miR-24 and miR-629

levels in comparison to DEN-treated STAT3^{f/f} mice (Figure 5B). These data show that suppression of the inflammatory response in vivo perturbs the HNF4 α circuit, suggesting that this circuit can be affected at any step.

Perturbation of the HNF4 α Circuit Has Therapeutic and Preventive Effects in Different Murine Liver Cancer Models

To further validate the in vivo significance of the HNF4 α circuit, we asked how perturbation of this circuit would affect tumor growth

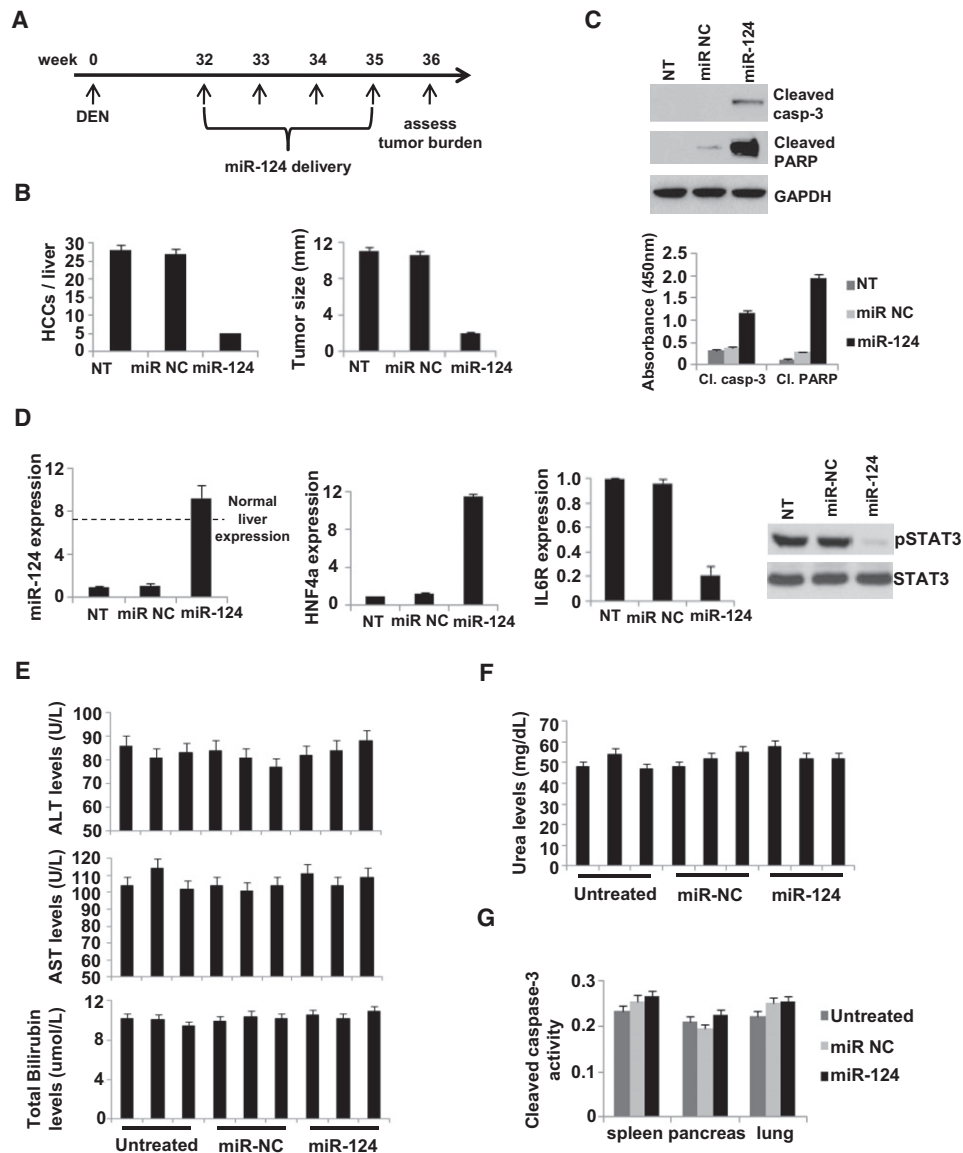


Figure 6. Modulation of the HNF4 α Circuit Prevents and Suppresses HCC Development in Mice

(A) Timeline of miR-124 therapeutic delivery experiment.

(B) Number of HCC tumors/liver and tumor size (mm³) (mean \pm SD) in nontreated (NT), miR-NC-, and miR-124-treated mice (week 36).

(C) Levels of cleaved PARP and caspase-3 in untreated, miR-NC-, and miR-124-treated mice (week 36) assessed by ELISA and western blot analyses.

(D) Evaluation of miR-124 levels, HNF4 α levels, and IL6R levels by real-time PCR and STAT3 phosphorylation status (Tyr705) by western blot in tumors derived from nontreated (NT), miR-NC-, and miR-124-treated mice (week 36). The data are shown as mean \pm SD.

(E and F) Levels of (E) alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and (F) urea were assessed in the serum of mice treated with 10 mg/kg miR-NC or miR-124 for 48 hr. Each bar represents a different mouse. The experiment was performed in triplicate, and the data show mean \pm SD.

(G) Cleaved caspase-3 activity (mean \pm SD) assessed by ELISA assays in tissues (spleen, pancreas, heart) derived from untreated, miR-NC-, or miR-124-treated mice.

in different HCC mouse models. Specifically, the inhibitory role of miR-124 on hepatocellular neoplastic transformation suggested the possibility that HCC-derived tumors could be eradicated efficiently by interference with the feedback loop on the level of miR-124. We found that miR-124 treatment suppressed HepG2 and SNU-449 xenograft tumor growth (Figure 5C) by reducing IL6R, miR-24, and miR-629 expression levels and significantly increasing HNF4 α expression (Figure 5D).

In addition to the subcutaneous HCC mouse model, we tested whether systemic administration of miR-124 is able to suppress HCC tumor growth in DEN-treated mice. According to our treatment protocol, miR-NC or miR-124 was systemically administered in DEN-treated mice on a weekly basis (first day of the week) for four cycles (weeks 32, 33, 34, and 35) (Figure 6A). On week 36, the mice were sacrificed, and we assessed the tumor burden. We found that miR-124 suppressed > 80% HCC tumor

growth and size (Figure 6B) through induction of apoptosis (Figure 6C), and actually, miR-124 administration resulted in restoration of physiological miR-124 expression, whereas miR-NC administration did not have any effect (Figure 6D). In addition, miR-124 delivery perturbed the HNF4 α circuit through upregulation of HNF4 α mRNA levels, IL6R suppression, and inhibition of STAT3 activation (phosphorylation). Importantly, we found that systemic delivery of miR-NC or miR-124 did not affect liver and kidney function (Figures 6E and 6F) and did not have any toxicity effects on essential organs (Figure 6G). These data demonstrate that miR-124 administration does not affect the physiology of mice through induction of cytotoxic effects.

In addition to therapeutic effects, we examined whether perturbation of the HNF4 α circuit can prevent HCC development in mice. We identified that miR-124 delivery restored the physiological levels of this microRNA in liver tumors, even 2 weeks postinjection (Figure S7A). According to these data, miR-124 was administered systemically on week 12 every 2 weeks until week 30, and at week 32, we assessed tumor burden (Figure S7B). We found that miR-124 delivery prevented efficiently HCC tumor growth in DEN-treated mice (Figure S7C), suggesting that the HNF4 α -miRNA inflammatory circuit is essential for HCC development in vivo.

The HNF4 α Regulatory Circuit Is Perturbed in Human HCC Tissues

We examined the expression levels of miR-24, IL6R, miR-124, and HNF4 α in total RNA extracted from 12 normal liver tissues and 45 hepatocellular carcinomas (HCCs). We found that HNF4 α and miR-124 were downregulated, whereas miR-24 and IL6R mRNA levels were increased in liver cancers relative to normal tissues (Figure 7A). In addition, immunohistochemical (IHC) analysis for HNF4 α and phosphorylated STAT3 and in situ hybridization for miR-124, miR-24, and miR-629 revealed that, in 13/30 (43.3%) of HCC tumors, the circuit is perturbed (Figure 7B).

Due to the fact that our in vitro data suggest that activation of an inflammatory response through suppression of HNF4 α levels is cell autonomous, we examined the activation of the inflammatory circuit in the absence of Kupffer cells. We tested expression levels of each member of the HNF4 α circuit in RNA samples derived from laser capture microdissected hepatocytes, which were negative for CD45 expression. Specifically, in all (8/8) human normal liver tissues, we found high HNF4 α and miR-124 levels and low IL6R, miR-24, and miR-629 levels. On the other hand, we identified that the HNF4 α circuit is perturbed (HNF4 α and miR-124 low levels; IL6R, miR-24, and miR-629) in 18/31 of human hepatocellular carcinomas (Figure 7C). Furthermore, in the same samples, we tested whether there is any correlation between the RNA expression levels of the different members of this circuit. We found an inverse correlation between HNF4 α and miR-24 or miR-629 levels, an inverse correlation between miR-124 and IL6R levels, and a positive correlation between HNF4 α and miR-124 levels (Figure 7D). Also, in the same human tissue samples, we examined IL6 and IL6R protein levels and STAT3 phosphorylation status and identified that the HCC samples ($n = 18$) with perturbed HNF4 α circuit have higher levels in comparison to the HCC

samples ($n = 13$) with nonperturbed HNF4 α circuit or normal liver tissues ($n = 8$) (Figure 7E).

Furthermore, we were interested in identifying whether the HNF4 α circuit is perturbed not only during liver cancer initiation, but also during liver cancer progression. Thus, we examined the mRNA expression levels of the different members of the circuit in different stages of HCC oncogenesis. We found that HNF4 α and miR-124 levels were decreased, whereas IL6R and miR-24 levels were increased, during HCC progression (Figure 8A). Interestingly, the activity of this circuit correlated to HCC grade (Figure 8B). Overall, these data strongly suggest that, in addition to tumor initiation, the HNF4 α -miRNA inflammatory feedback circuit is important for the progression of human cancer.

DISCUSSION

An HNF4 α Circuit Is Essential for the Transformation of Immortalized Hepatocytes

Our data reveal the dynamics of a complex molecular self-reinforcing circuit that involves HNF4 α , miR-124, IL6R, STAT3, and miR-24/miR-629 in the regulation of hepatocellular transformation and liver cancer (Figure 7F). The first component of the circuit links HNF4 α to STAT3 activation, with HNF4 α controlling IL6R expression through transcriptional regulation of miR-124. Although miR-124 has been identified as a cancer-associated tumor suppressive microRNA (Lujambio et al., 2007), its regulation and mode of action has been elusive. Here, we show that HNF4 α binding and transcriptional regulation of miR-124 are comparable to the bona fide HNF4 α target ApoCIII (Kardassios et al., 1997; Ladas et al., 1992). The second component of the circuit connects STAT3 activity to HNF4 α expression via regulation of miR-24 and miR-629. Perturbations of the STAT3-HNF4 α axis interfere with processes that govern hepatic transformation and oncogenesis, mechanistically linking inflammation and liver cancer.

An Epigenetic Switch Regulates Hepatocyte Transformation

The main characteristic of the HNF4 α feedback circuit is that it transforms immortalized human hepatocytes by converting a transient signal (e.g., acute HNF4 α inhibition) into a stable signal. Overexpression of any positive factor (miR-24, miR-629) or inhibition of any negative factor (HNF4 α , miR-124) transforms immortalized hepatocytes, indicating that the loop can be affected at any step. Thus, the initiating event in different HCC mouse models and patients can be different. It is not necessary that the loop begins with reduction of HNF4 α . According to our data, suppression of HNF4 α expression is the first event in DEN-treated mice, followed by perturbation of the other members of the loop. In other scenarios, the IL6-STAT3 axis may activate the loop by different extracellular stimuli. Specifically, secreted IL6 from different immune cells in the tumor microenvironment, including Kupffer cells, could initiate this axis. For example, recent studies show that IL-22, a cytokine secreted by Th17 cells, controls hepatocellular oncogenesis via upregulation of STAT3 activity (Jiang et al., 2011), and hepatitis C viral infection can promote STAT3 activation (Tacke et al., 2011). Interestingly, miR-124 has been found epigenetically

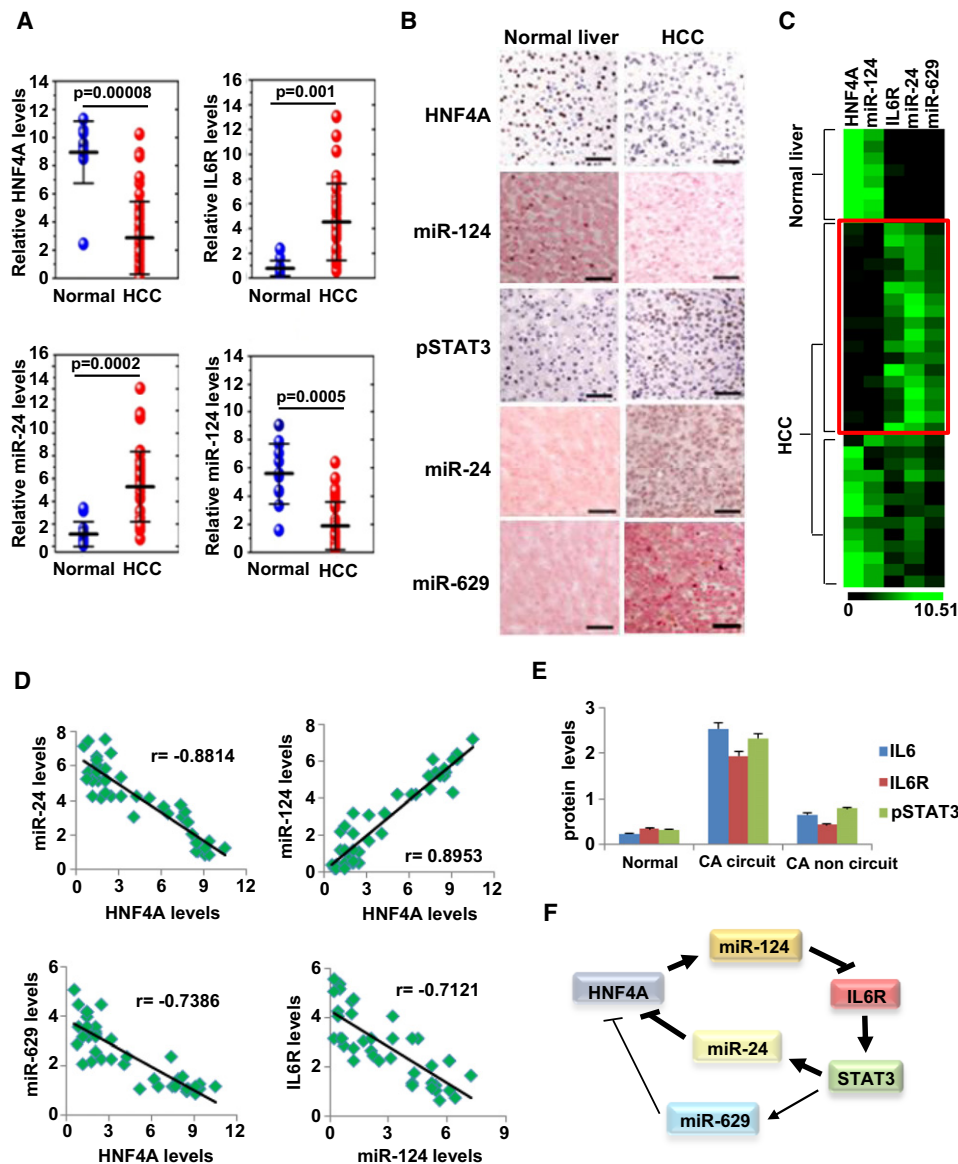


Figure 7. HNF4 α Circuit Is Perturbed in Human Hepatocellular Carcinomas

(A) Assessment of HNF4 α , IL6R, miR-24, and miR-124 levels (mean \pm SD) by real-time PCR analysis in total RNAs derived from 12 normal liver tissues and 45 hepatocellular carcinomas.

(B) Immunohistochemistry for HNF4 α , pSTAT3, and in situ hybridization for miR-124 and miR-24 in FFPE sections of hepatocellular carcinomas and normal liver tissues. Sections were subjected to immunohistochemistry for HNF4 α (DAB staining, brown) and phospho-STAT3 (Tyr705) (DAB staining) and counterstained with hematoxylin (blue) and in situ hybridization for miR-124, miR-24, and miR-629 and counterstained with nuclear fast red. Scale bar, 100 μ m.

(C) Heatmap representation of HNF4 α , IL6R, miR-24, and miR-124 levels assessed by real-time PCR (mean \pm SD) in tissue-microdissected FFPE sections of eight normal liver tissues and 31 hepatocellular carcinomas.

(D) Correlation between the expression levels of different members of the HNF4 α circuit (same samples as in Figure 7C). Each data point represents an individual liver tissue sample, and a correlation coefficient (r) is shown.

(E) Levels of IL6, IL6R, and pSTAT3 (Tyr705) assessed by ELISA in eight normal liver tissues, 31 hepatocellular carcinomas (18 tissues with activation of the HNF4 α circuit [CA circuit] and 13 liver cancer tissues without activation of the HNF4 α circuit [CA noncircuit]). The data are presented as mean \pm SD of three independent experiments.

(F) Schematic representation of the proposed HNF4 α feedback circuit in hepatocellular oncogenesis.

silenced through tumor-specific methylation both in human HCC cell lines and tissues (Furuta et al., 2010), and therefore miR-124 downregulation may be the first event that triggers hepatic carcinogenesis. Together, all of these data suggest that the initial

event that activates this circuit could differ from patient to patient.

Due to the fact that the epigenetic switch in immortalized hepatocytes occurs within a few days, it is extremely unlikely

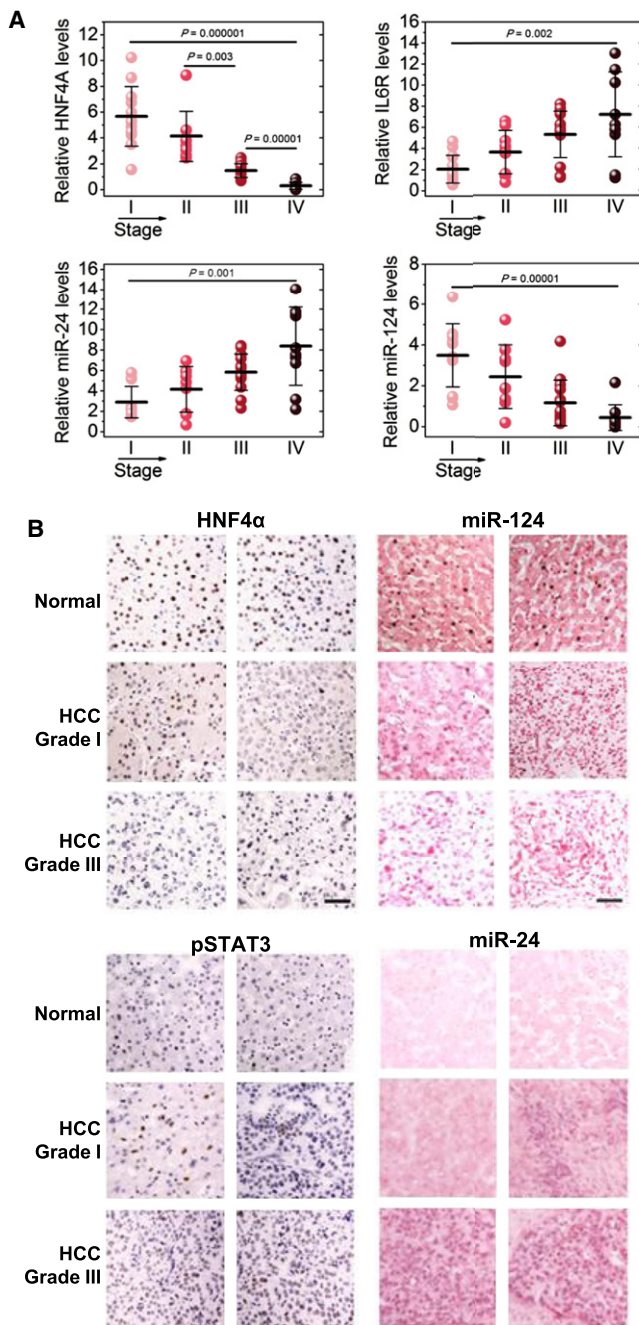


Figure 8. HNF4 α Circuit Is Perturbed during HCC Progression

(A) Assessment of HNF4 α , IL6R, miR-24, and miR-124 levels in total RNAs derived from 45 hepatocellular carcinomas, according to their tumor stage. The experiments have been performed in triplicate, and data are shown as mean \pm SD.

(B) HCC sections were subjected to immunohistochemistry for HNF4 α and phospho-STAT3 (Tyr705) (DAB staining, brown) and counterstained with hematoxylin (blue) and in situ hybridization for miR-124 and miR-24 and counterstained with nuclear fast red. Representative pictures are shown from normal, HCC grade I, and HCC grade III tissues. Scale bar, 50 μ m.

to involve changes in the DNA sequence, which is consistent with the definition of a true epigenetic switch (Ptashne, 2009). This notion of a self-reinforcing feedback loop that controls hepatocellular transformation comes in line with our previous observation of an epigenetic switch that mediated transformation of immortalized mammary epithelial (MCF10A) cells to a stably transformed cell (Iliopoulos et al., 2009, 2010). Furthermore, the identification of an epigenetic switch in hepatocellular transformation indicates that it is not a rare mechanism involved in cellular transformation and supports the possibility that cancer cells of diverse developmental origin might share a common mechanism for the establishment of the transformed state.

Our data also suggest that microRNA transcription factor regulatory circuits mediate epigenetic switches that induce transformation of immortalized cells. Recent reports posit that transcriptional (TFs) and nontranscriptional elements (microRNAs) may cooperate to tune gene expression in various biological processes (Chen et al., 1994; Gerstein et al., 2010; Martinez et al., 2008), including oncogenesis (Fabbri et al., 2011; Kent et al., 2010). Various network motifs have been proposed, but miRNA-TF feedforward and feedback loops predominate. For example, a feedforward regulatory circuit (KRAS-miR-143/miR-145) plays an essential role in pancreatic tumorigenesis (Kent et al., 2010). Taken together, these observations demonstrate that transcription factors participate in similar circuits that regulate induction and maintenance of stable transformation programs, suggesting that use of analogous regulatory loops may be a widespread property of oncogenic processes.

Role of HNF4 α and Its Downstream Effectors in Hepatocellular Oncogenesis

HNF4 α has long been considered a key transcription factor during liver embryonic development (Kyrnizi et al., 2006; Parviz et al., 2003). In the adult liver, HNF4 α is expressed at high levels and binds to the promoter of 12% of genes expressed (Odom et al., 2004). Nevertheless, HNF4 α 's role in hepatocellular cancer and the mechanisms involved are far from clear. It has been shown that HNF4 α is upregulated in human hepatocellular carcinoma (Xu et al., 2001) and, on the other hand, impedes the formation of liver tumors in mice by inducing differentiation of malignant cells—including cancer stem cells—into mature hepatocytes (Yin et al., 2008). Recent findings that the Wnt/ β -catenin pathway interacts with HNF4 α in intestinal epithelial cells (Cattin et al., 2009) and hepatocytes (Colletti et al., 2009) strengthen the notion that HNF4 α acts as a tumor suppressor gene in both cancer types. Our study refines the repressive role of HNF4 α in hepatic neoplasia, suggesting that HNF4 α inhibition mediates an epigenetic switch that is essential for the transformation of immortalized hepatocytes.

Inflammation is one of the downstream mechanisms linking HNF4 α to hepatocellular carcinogenesis. The protective action of HNF4 α against inflammatory bowel diseases (Ahn et al., 2008; Darsigny et al., 2010) and the potential associations between the HNF4A locus and ulcerative colitis (Barrett et al., 2009) raise the possibility that this multifaceted transcription factor is a potent mediator of inflammatory responses (Marcil et al., 2010). Several studies have identified STAT3 as an oncogenic transcription factor activated by inflammatory responses

(Bromberg et al., 1999; Grivennikov et al., 2009; Iliopoulos et al., 2009; Iliopoulos et al., 2010), and IL6 is known to directly activate STAT3 (Zhong et al., 1994). STAT3 activity has been correlated with poor prognosis in HCC patients (Calvisi et al., 2006), and STAT3 inhibitors inhibit the growth of several human cancers (Hedvat et al., 2009), including HCC development and growth in mice (He et al., 2010).

As genetic alterations that result in constitutive STAT3 activation in hepatocytes only cause benign hepatic adenomas unless combined with oncogenic mutations (Rebouissou et al., 2009), it will be important to discover the parameters that distinguish primary hepatocytes from nontransformed immortalized cells. Although a transient inflammatory signal is insufficient to trigger such an epigenetic switch in normal hepatocytes, our *in vivo* data suggest that the epigenetic switch described here is relevant to human cancer. The epigenetic switch requires that a transient inflammatory response is converted to a chronic inflammatory response, with no resolution phase but continuous enhancement of the inflammatory signal. Thus, the results presented here provide a paradigm in which a key step in transformation involves an epigenetic switch in response to an inflammatory signal, as opposed to a mutational change in a tumor suppressor or oncogene. In support of this idea, recent data suggest that chronic activation of the IL6-STAT3 axis contributes to the transformation of hepatocytes that have acquired oncogenic mutations upon exposure to environmental and dietary carcinogens (Park et al., 2010).

Therapeutic and Preventive Effects of miR-124 Delivery in Hepatocellular Carcinogenesis

We show that miR-124 administration restored miR-124 expression to physiological levels in the liver, inhibiting and preventing DEN-induced hepatocellular carcinogenesis in mice. Previous studies have aimed to suppress microRNA expression in animal models through delivery of antagomirs or locked nucleic acid (LNA) oligomers (Elmén et al., 2008; Esau et al., 2006). Few studies have investigated the therapeutic delivery of microRNAs *in vivo*. A recent study has shown that restoration of miR-26a expression levels by an adeno-associated virus (AAV) suppresses liver tumorigenesis in liver-specific MYC transgenic mice (Kota et al., 2009) without any cytotoxic effects.

Our data suggest that systemic delivery of miR-124 may be a clinically viable anticancer therapeutic approach for liver cancer. Delivery of microRNAs in the liver is more efficient in comparison to other tissues, and a recent study revealed that delivery of the antisense-microRNA-122 suppressed hepatitis C viremia in primates, with no evidence of viral resistance or side effects (Lanford et al., 2010), leading to the initiation of phase I clinical trials in HCV-infected patients. This work lays the ground for testing whether miR-124 can also exert tumor-suppressive effects in human liver cancers. Together, our findings elucidate a molecular mechanism that is responsible for the initiation and maintenance of the hepatocyte-transformed phenotype, which enhances our understanding of liver cancer pathogenesis and provides a microRNA therapeutic strategy for prevention and treatment of liver cancer. Though we have identified a novel molecular circuit that is essential for the transformation of hepatocytes and is found to be perturbed in

different HCC models and in human hepatocellular carcinomas, significant work remains to identify the driver signaling pathways involved in hepatocellular carcinogenesis.

EXPERIMENTAL PROCEDURES

Extensive details for all experimental procedures are provided in the [Extended Experimental Procedures](#).

Cell Culture

Human nontransformed immortalized hepatocytes (IMH1, IMH2) were purchased from ATCC (cat no. CRL-4020) and from Xenotech LLC (cat. no. IFH15), respectively. Detailed description of the origin of these immortalized hepatocytes and their culture conditions can be found in the [Extended Experimental Procedures](#). In addition, human liver cancer cell lines (HepG2, Hep3B, SNU-449, SNU-398, and SNU-387) were purchased from ATCC. Human liver cancer cell lines HepG2 and Hep3B were maintained in DMEM medium (GIBCO) supplemented with 10% FBS and 10 units/ml penicillin and 100 μ g/ml streptomycin. SNU-449, SNU-398, and SNU-387 were maintained in RPMI-1640 medium (GIBCO) supplemented with 10% FBS and 10 units/ml penicillin and 100 μ g/ml streptomycin.

MicroRNA Library HNF4 α Screening

A microRNA library consisting of 317 microRNA mimics and two microRNA negative control mimics (100 nM) (Dharmacon Inc) was transfected in HepG2 cells plated in 96-well plates. At 24 hr posttransfection, the cells were transfected with a firefly luciferase vector harboring the 3'UTR of HNF4 α for 24 hr, and the luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, WI, USA). MicroRNAs that inhibited > 75% the luciferase activity of HNF4 α were considered as positive hits. Detailed experimental description can be found in the [Extended Experimental Procedures](#).

TUNEL Assay

Apoptosis was determined using the DeadEnd Fluorometric TUNEL System (G3250, Promega), as previously described (Polytarchou et al., 2008).

Real-Time PCR Analysis

RNA purified from IMH, HepG2, Hep3B, SNU-449, SNU-398, SNU-387, and SNU-475 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. MicroRNA expression levels were tested using the Exiqon PCR Primer Sets, according to the manufacturer's instructions (Exiqon Inc, Denmark). Primer sequences can be found in the [Extended Experimental Procedures](#).

Identification of Transcription Factor Sites in MicroRNA Regulatory Areas

The Lever and PhyICRM algorithms have been used to identify STAT3 and HNF4 α binding motifs in an area 5 kb upstream and 2 kb downstream of microRNAs. A detailed description of this method has been included in the [Extended Experimental Procedures](#).

Mouse Experiments

All of the experiments in xenografts and DEN-treated mice are described analytically in the [Extended Experimental Procedures](#).

RNA Expression Studies from Patient 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 HNF4 α were analyzed by real-time RT-PCR in all of the tissue described above. Each sample was run in triplicate, and the data represent the mean \pm SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.cell.2011.10.043.

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