

A miRNA-mediated attenuation of hepatocarcinogenesis in both hepatocytes and Kupffer cells

Yan Tian,^{1,2,6} Mingfeng Zhang,^{2,6} Mingjie Fan,² Haixia Xu,¹ Shunquan Wu,² Sailan Zou,¹ Yangmeng Wang,² Dongmei Tang,¹ Chunyan Zhang,³ Weidong Han,^{2,4} Hua Yu,^{3,5} Xianghui Fu,^{1,2} and Wendong Huang^{2,5}

¹Division of Endocrinology and Metabolism, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Chengdu, 610041 Sichuan, China; ²Department of Diabetes Complications and Metabolism, Arthur Riggs Diabetes and Metabolism Research Institute, Beckman Research Institute, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA; ³Department of Immuno-oncology, Beckman Research Institute, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA; ⁴Department of Medical Oncology, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, 3 East Qingchun Road, Hangzhou, Zhejiang 310016, China; ⁵Graduate School of Biological Science, City of Hope National Medical Center, Duarte, CA 91010, USA

MicroRNAs (miRNAs) are small noncoding RNAs that regulate a variety of physiological and pathological functions. miR-26a is one of the many miRNAs that have been identified as regulators of cancer development and as potential anticancer drug targets. However, the specific cellular and molecular mechanisms by which miR-26a attenuates hepatocarcinogenesis are still elusive. Here, we interrogated mouse models with miR-26a cell-specific overexpression in either hepatocytes or myeloid cells to show that miR-26a strongly attenuated the chemical-induced hepatocellular carcinoma (HCC). miR-26a overexpression broadly inhibited the inflammatory response in both hepatocytes and macrophages by decreasing several key oncogenic signaling pathways in HCC promotion. These findings thus reveal new insights into a concerted role of miR-26a in both hepatocytes and Kupffer cells to suppress hepatocarcinogenesis, thereby highlighting the potential use of miR-26a mimetics as potential approaches for the prevention and treatment of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC), the most common form of liver cancer, remains the third leading cause of cancer mortality. In contrast to the decreasing mortality rates observed for many other types of cancers, overall death from liver cancer has significantly increased in the United States over the past 20 years.^{1,2} Major hurdles in developing effective therapies for HCC include its considerable intratumoral heterogeneity and the absence of well-established, broadly targetable oncogene addiction (presence of a single dominant oncogene responsible for growth and survival). Current evidence indicates that during hepatocarcinogenesis, two main pathogenic mechanisms prevail: (1) cirrhosis associated with pathological chronic liver inflammation after tissue damage caused by hepatitis B virus (HBV), HCV, toxins (e.g., alcohol, aflatoxin), or metabolic influences; and (2) mutations occurring in single or multiple oncogenes or tumor suppressor

genes.^{3,4} Both mechanisms have been linked with alterations in several important cellular signaling pathways contributing to uncontrolled cell proliferation and growth. Furthermore, targeted therapies, such as small-molecule receptor tyrosine kinase inhibitors or monoclonal anti-growth factor (receptor) antibodies, still have very limited therapeutic effect for patients with HCC. For example, sorafenib, which was initially developed as an inhibitor of Raf-kinase, showed a broad activity against several tyrosine kinases, including the angiogenic factors vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), and has been demonstrated to have limited survival benefit, with a very low rate of tumor response (2%–3%).^{5,6} The major unresolved issues that hinder patient success in HCC are the following: lack of mechanistic knowledge, lack of high-quality diagnostic markers, and lack of effective therapeutics. Therefore, better understanding of the pathogenetic mechanisms of HCC and developing novel approaches to predict or treat HCC are urgently needed.

MicroRNAs (miRNAs) constitute a large class of phylogenetically conserved, single-stranded RNA molecules of 19–25 nt that are involved in post-transcriptional gene silencing by binding to specific regions of target mRNA transcripts and either destabilizing the target mRNA transcript, blocking its translation, or both.⁷ A growing body

Received 13 March 2022; accepted 23 August 2022;
<https://doi.org/10.1016/j.omtn.2022.08.036>.

⁶These authors contributed equally

Correspondence: Xianghui Fu, Division of Endocrinology and Metabolism, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, China.

E-mail: xfu@scu.edu.cn

Correspondence: Wendong Huang, PhD, Department of Diabetes Complications and Metabolism, Arthur Riggs Diabetes and Metabolism Research Institute, Beckman Research Institute, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010, USA.

E-mail: whuang@coh.org



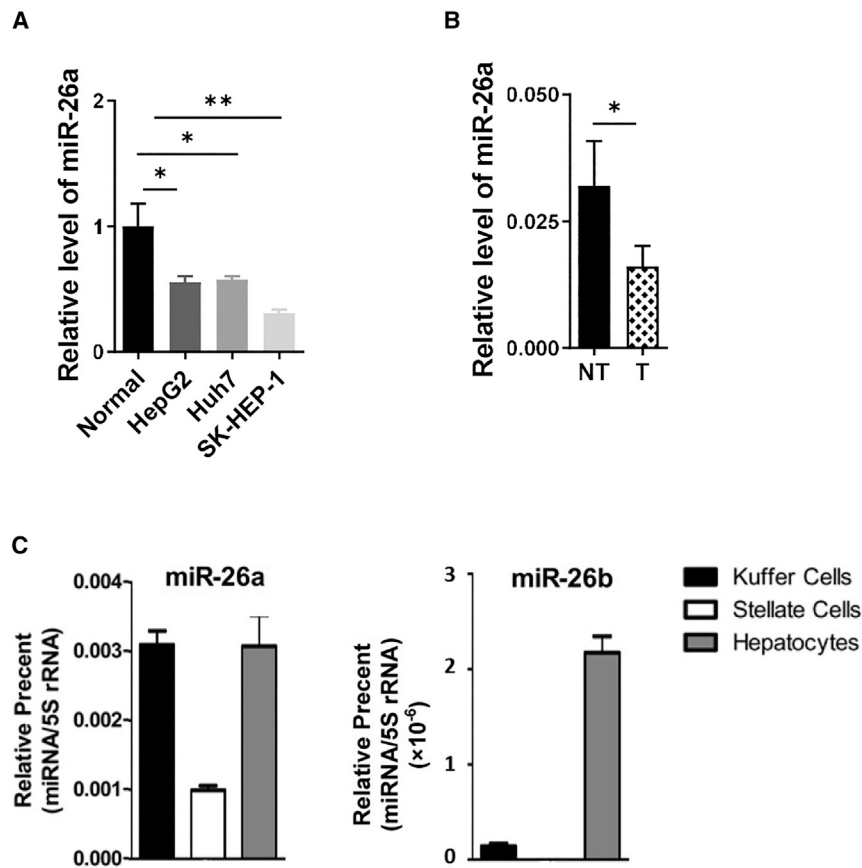


Figure 1. miR-26a is equally expressed in hepatocytes and Kupffer cells

(A) qRT-PCR analysis of miR-26a expression in 1 normal and 2 HCC cell lines (HepG2 and Huh7), as well as SK-HEP-1 cells. (B) qRT-PCR analysis of miR-26a expression in tumor (T) and nontumor (NT) from aged FXR^{-/-} mice. (C) qRT-PCR analysis of miR-26a expression in primary Kupffer cells, stellate cells, and hepatocytes isolated from C57BL/6 mice. The expression levels of miRNAs were normalized to those of 5S RNA. *p < 0.05.

miR-26a overexpression. Both cell-type-specific miR-26a transgenic mice showed strongly reduced susceptibility to chemical-induced HCC. The results thus provide mechanistic insights into a concerted role of miR-26a in both hepatocytes and Kupffer cells to attenuate hepatocarcinogenesis.

RESULTS

miR-26a is equally expressed in hepatocytes and Kupffer cells in the liver

To identify miRNAs involved in hepatic cell proliferation and HCC development, we previously developed a miRNA dot blot array by spotting antisense miRNA oligonucleotides onto N⁺ membranes. Endogenous liver miRNA labeling was performed using the mirVana

miRNA labeling kit with minor modifications (Ambion).²² We detected the expression of ~80 miRNAs in the liver. Substantial differences in expression levels were observed: a few miRNAs, such as miR-26a and miR-122, displayed high expression in liver tissue, whereas most of the remaining miRNAs showed modest or weak expression.²² We compared the expression of miR-26a in a normal liver cell compared to three HCC cell lines (HepG2, Huh7, and SK-HEP-1). The results showed a significantly reduced expression of miR-26a in all three HCC cancer cells (Figure 1A). We then investigated whether miR-26a is involved in HCC. We used the Farnesoid X receptor (FXR) knockout mouse model for these experiments because we have shown that aged (≥ 12 months) FXR knockout mice spontaneously develop HCC and pathologically mimic human HCC progression.^{23,24} The expression of miR-26a was dramatically reduced in tumors (T) as compared to nontumors (NT) from aged FXR knockout mice (Figure 1B), and this result is consistent with the previous observations that miR-26a is significantly downregulated in human HCC.^{20,21,25} Thus, these lines of evidence suggest that miR-26a may play a tumor-suppressor role in HCC development. To further elucidate the functions of miR-26a, we profiled its expression pattern in various tissues, as well as different cell types in the liver. Human miR-26a was expressed at high levels in nearly all of the tissues we examined.¹⁸ MiR-26a was also expressed broadly in various mouse liver cell types and was equally expressed in hepatocytes and Kupffer

of evidence indicates that miRNAs are frequently deregulated in human malignancies and function as either oncogenes or tumor suppressor genes.⁸ In recent years, the potential for miRNA biology to clarify both the molecular pathogenesis of cancer and the inherent complexities in translating its biology to the clinic has gained much attention.⁹

Increasing evidence suggests that miRNAs are promising therapeutic targets.^{10–13} We and others have shown that miR-26a plays critical roles in a variety of physiological and pathological processes, including pancreatic cell differentiation,^{14,15} liver disorder,^{15,16} and glucose/lipid metabolism.^{17,18} We have also previously shown that miR-26a acts as a modulator of miRNA maturation in cancer.¹⁹ There is also clinical evidence for the tumor-suppressive roles of miR-26a in HCC.^{20,21} However, the exact cellular and molecular mechanisms by which miR-26a attenuates HCC are still elusive. Although therapeutic modulation of miRNAs, including miR-26a, is an emerging field, the ability to inhibit groups of genes that regulate cancer development and the ability to target tumor cells as well as the tumor microenvironment has tremendous potential for HCC treatment.

In the present study, we determined the cellular and molecular mechanisms by which miR-26a attenuates hepatocarcinogenesis using transgenic mice with either hepatocyte- or myeloid cell-specific

cells (Figure 1C, left panel). In contrast, miR-26b was expressed mainly in hepatocytes, but at a lower level (Figure 1C, right panel). Because both hepatocytes and Kupffer cells are critical for the development of HCC, the relative contribution of miR-26a in each cell type during hepatocarcinogenesis remains to be determined.

Mice with overexpression of miR-26a in hepatocytes (miR-26a^H) are resistant to *N*-nitrosodiethylamine (DEN)-induced hepatocarcinogenesis

We previously established and used DEN-induced HCC models to delineate the function and mechanism of potential regulators in hepatocarcinogenesis.^{23,26} The advantage of chemical-induced models, such as the DEN model, is their similarity to the injury-fibrosis-malignancy cycle seen in humans, making them the favored models for HCC research. To determine the effect of miR-26a overexpression in hepatocytes on HCC development, we used our previously established HCC model to compare HCC development in wild-type (WT) and miR-26a transgenic mice.²⁷ This approach involves an initial injection of DEN, followed by sequential treatments with 1,4 bis[2-(3,5-dichloropyridyl)] benzene (TCPOBOP). This protocol induces liver tumors in all mice after approximately 4–5 months. As shown in Figures 2A–2C, both sexes of miR-26a^H mice showed a greatly reduced number of total and large tumors compared to those of control WT littermates. We also used a second HCC model: 15-day-old miR-26a^H and their littermate WT controls were subjected to DEN + TCPOBOP-induced hepatocarcinogenesis (Figures 2D–2F). The results showed a similar outcome that both sexes of mice with overexpression of miR-26a in hepatocytes (miR-26a^H) are resistant to DEN-induced hepatocarcinogenesis. Finally, we used a third HCC model that male mice received one DEN injection when they were 15 days of age; 8 months later, miR-26a^H mice developed much less HCC compared to the WT control mice (Figure 2G). We measured the expression levels of mature miR-26a and Pre-miR-26a in either nontumor or tumor liver tissues from WT and miR-26a^H mice. The results show an overexpression of miR-26a in either nontumor or tumor liver tissues from miR-26a^H but not WT mice, indicating that hepatic miR-26a expression remained stable during DEN-induced hepatocarcinogenesis (Figure S2). Consistently, expression of mature miR-26a, but not pre-miR-26a-1, was significantly reduced compared to that in nontumor liver tissues.

Overexpression of miR-26a in hepatocytes suppresses DEN-induced liver inflammation

Inflammation, such as chemical-induced liver inflammation, is well known to promote HCC development. To determine whether overexpression of miR-26a in hepatocytes suppresses DEN-induced inflammation, we measured the expression levels of several well-known cytokines involved in inflammatory responses in tumor and nontumor liver tissues. The results indicated that overexpression of miR-26a in hepatocytes significantly suppressed the expression of cytokines promoting liver inflammation in nontumor tissues, including interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α). However, inducible nitric oxide synthase (iNOS) is lower only in liver tumors

(Figure 3A). In contrast, the cytokines (mainly IL-10, macrophage galactose-type lectin 1 [MGL1]) that suppress inflammation were significantly upregulated in miR-26a^H liver tumor tissues compared to the control littermates (Figure 3B). We also conducted western blotting analysis to examine several major pathways regulating liver inflammation in DEN-induced liver tumors. As expected, generally these signaling pathways, including Stat3, p65, and I κ B α are more activated in tumor (T) compared to nontumor tissues (NT). To our surprise, compared to the WT controls, the overexpression of miR-26a in hepatocytes did not significantly change the activation of these signaling pathways (Figure 3C). These results not only confirmed a tumor suppressor role of miR-26a in hepatocytes but also imply that other cells in addition to hepatocytes may play roles during DEN-induced hepatocarcinogenesis.

Mice with overexpression of miR-26a in myeloid cells (miR-26a^{M/N}) are resistant to DEN-induced hepatocarcinogenesis

MiR-26a has been reported to regulate macrophage functions.^{28–30} To determine the impact of miR-26a in macrophages on hepatocarcinogenesis, we crossed LysM-Cre mice with miR-26aTg mice and generated a new mouse line that specifically overexpressed miR-26a in myeloid lineage cells (MiR26a^{M/N}). As shown in Figure S1C, the expression of miR-26a in intraperitoneal macrophages was approximately six times higher in MiR26a^{M/N} mice compared to their WT littermates. MiR26a^{M/N} mice and their WT control littermates were then treated with DEN, followed by six sequential treatments with TCPOBOP (Figure 4A). The tumor burden was assessed 20 weeks after DEN injection. As shown in Figure 4B, similar to the results obtained with miR-26a^H mice, overexpression of miR-26a in myeloid cells significantly reduced the DEN-induced hepatocarcinogenesis. The number of HCC in MiR26a^{M/N} mice was much lower compared to that in WT control mice (Figure 4C, left panel), and the maximum tumor size was also significantly smaller in miR-26a^{M/N} mice compared to that in WT control mice (Figure 4C, right panel). These results revealed that specific overexpression of miR-26a in myeloid cells is sufficient to protect mice from DEN-induced hepatocarcinogenesis.

Overexpression of miR-26a in myeloid cells reduces chemical-induced acute inflammation *in vivo*

To further understand how myeloid-specific miR-26a overexpression suppresses HCC tumorigenesis, we examined the chemical-induced acute inflammation *in vivo*. We subjected both miR-26a^H and miR-26a^{M/N} mice to a single dosage of DEN injection (100 mg/kg), and 4 h later, they were euthanized to collect liver tissues to measure the expression of several key cytokines involved in inflammatory responses. The results showed a trend of reduced expression levels of three major cytokines (IL-6, TNF- α , and IL-1 β) in miR-26a^H livers compared to the WT control (Figure 5A). Strikingly, in miR-26a^{M/N} mice, all of these cytokines displayed significantly lower expression levels compared to the WT control, indicating a stronger effect of miR-26a overexpression in myeloid cells to inhibit inflammation compared to that in hepatocytes (Figure 5B). The results were confirmed by ELISA assay to measure protein levels (Figure S3). To

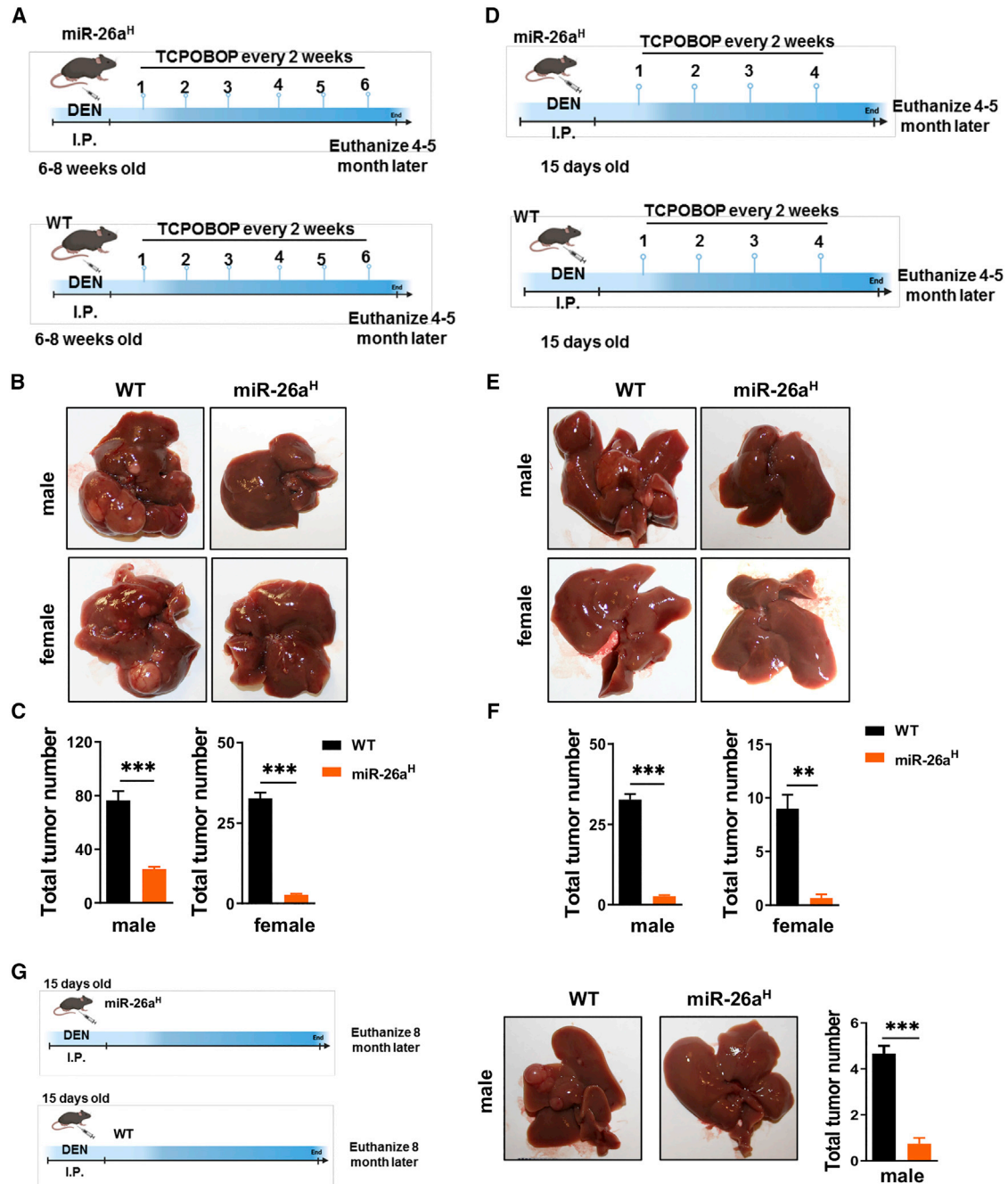


Figure 2. Hepatic overexpression of miR-26a in mice-attenuated DEN-induced hepatocarcinogenesis

(A–C) Six- to 8-week-old hepatic overexpression of miR-26a transgenic mice (miR-26a^H) and their littermate controls (WT) were subjected to DEN + TCPOBOP-induced hepatocarcinogenesis (male: n = 6–7 mice/group; female: n = 4 mice/group). (A) Schematic showing the experimental model and the treatment strategy. (B) Representative images of liver tumorigenesis. (C) Number of visible tumor foci in mouse livers. (D–F) Fifteen-day-old miR-26a^H and their littermate WT controls were subjected to g DEN + TCPOBOP-induced hepatocarcinogenesis (n = 3–4 mice/group). (D) Schematic showing the experimental model and the treatment strategy. (E) Representative images of liver tumors. (F) Number of visible tumor foci in mouse livers. (G) Fifteen-day-old miR-26a^H and their littermate WT controls were subjected to DEN-induced hepatocarcinogenesis (n = 4–6 mice/group). *p < 0.05; **p < 0.01; ***p < 0.005.

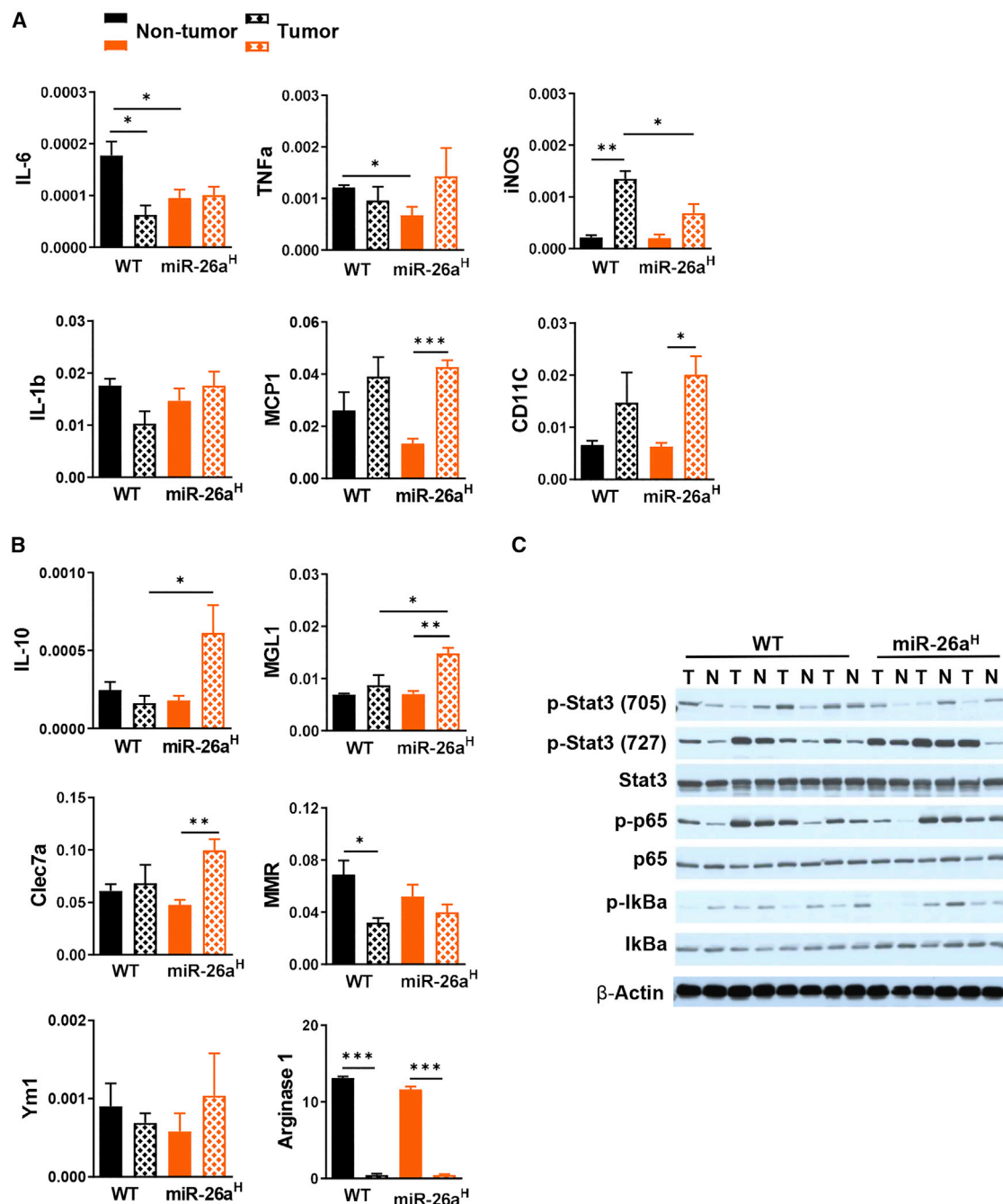


Figure 3. Hepatic overexpression of miR-26a reduced inflammation in DEN-induced hepatocarcinogenesis

(A and B) qRT-PCR analysis of expression of genes regulating liver inflammation in tumors and nontumor liver tissues. (C) Representative western blot analysis of major pathways involved in regulating liver inflammation in tumor (T) and nontumor (N) tissues from WT and miR-26a^H mice. mRNA expression levels were normalized to those of β-actin. **p* < 0.05; ***p* < 0.01; ****p* < 0.005.

further confirm these observations, we switched to another pro-inflammatory stimulant: lipopolysaccharides (LPS) (20 mg/kg body weight) injection. We observed a similarly significant reduction in IL-6 expression levels in miR-26a^{M/N} but not miR-26a^H mice (Fig-

ure 5C). More interestingly, we observed a significantly reduced phosphorylated STAT3 at Tyr 705 in miR-26a^{M/N} livers compared to the WT controls (Figure 5D). These results revealed that miR-26a overexpression in myeloid cells strongly reduced the acute induction of

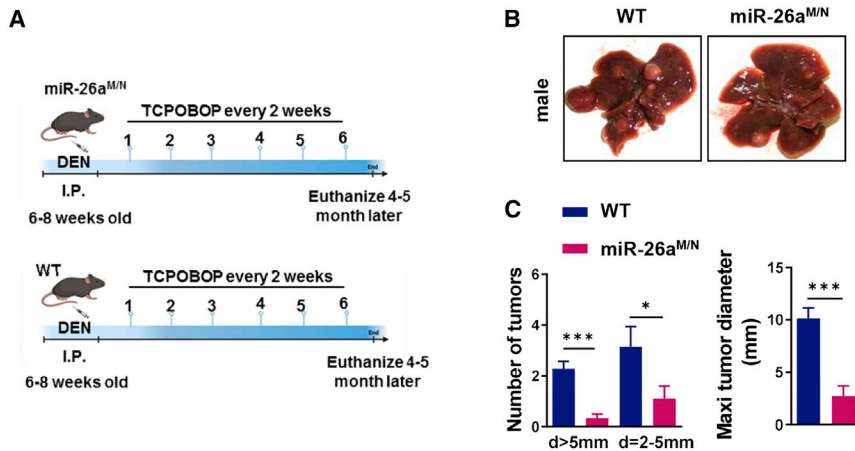


Figure 4. Myeloid-specific overexpression of miR-26a in mice-attenuated DEN-induced hepatocarcinogenesis

(A–C) Six- to 8-week-old myeloid-specific overexpression of miR-26a transgenic mice (miR-26a^{M/N}) and their littermate controls (WT) were subjected to DEN + TCPOBOP-induced hepatocarcinogenesis ((n = 4–6 mice/group)). (A) Schematic showing the experimental model and the treatment strategy. (B) Representative images of liver tumors. (C) Number of visible liver tumors (left panel) and the maximum size of liver tumors (right panel). *p < 0.05; ***p < 0.005.

cytokines promoting inflammation and STAT3 activation upon DEN-induced liver damage or LPS treatment.

miR-26a overexpression decreases expression of proinflammatory cytokines and reduces nuclear factor κ B (NF- κ B) activation in macrophages

Kupffer cells are liver resident macrophages that are known to produce cytokines promoting inflammation through NF- κ B activation.⁴ To determine a direct role of miR-26a in suppressing the inflammatory response in macrophages, we first transfected the pre-miR-26a or a negative control (NC) into RAW264.7 cells and J774A.1 cells, followed with LPS (10 ng/mL) treatment *in vitro* for different time points. The expressions of several cytokine genes were measured. The results showed that, compared to NC, pre-miR-26a significantly suppressed LPS-induced IL-6 and TNF- α expression in RAW264.7 cells (Figure 6A) and IL-1 β expression in J774A.1 cells (Figures S4 and S5A). We then isolated bone marrow-derived macrophages (BMDMs) from WT mice and the cells then transfected the pre-miR-26a or an NC, followed by LPS (10 ng/mL) treatment *in vitro* for 3 or 6 h. The expressions of cytokine genes were then measured. The results showed that pre-miR-26a significantly suppressed LPS-induced IL-6 at 6 h and IL-1 β at 3 h compared with BMDMs treated with NC (Figures 6B and S4). Next, we evaluated the activation of NF- κ B by LPS using western blot analysis. As expected, overexpression of miR-26a in macrophages repressed LPS-induced phosphorylation of p-65 (Figures 6C and S5B). Consistently, the nuclear p65 protein levels were also significantly reduced compared to that in the control cells (Figure 6D). These data indicated that miR-26a negatively regulated the NF- κ B signaling pathway, resulting in lower levels of proinflammatory cytokine expression.

miR-26a directly targets several genes regulating liver inflammation

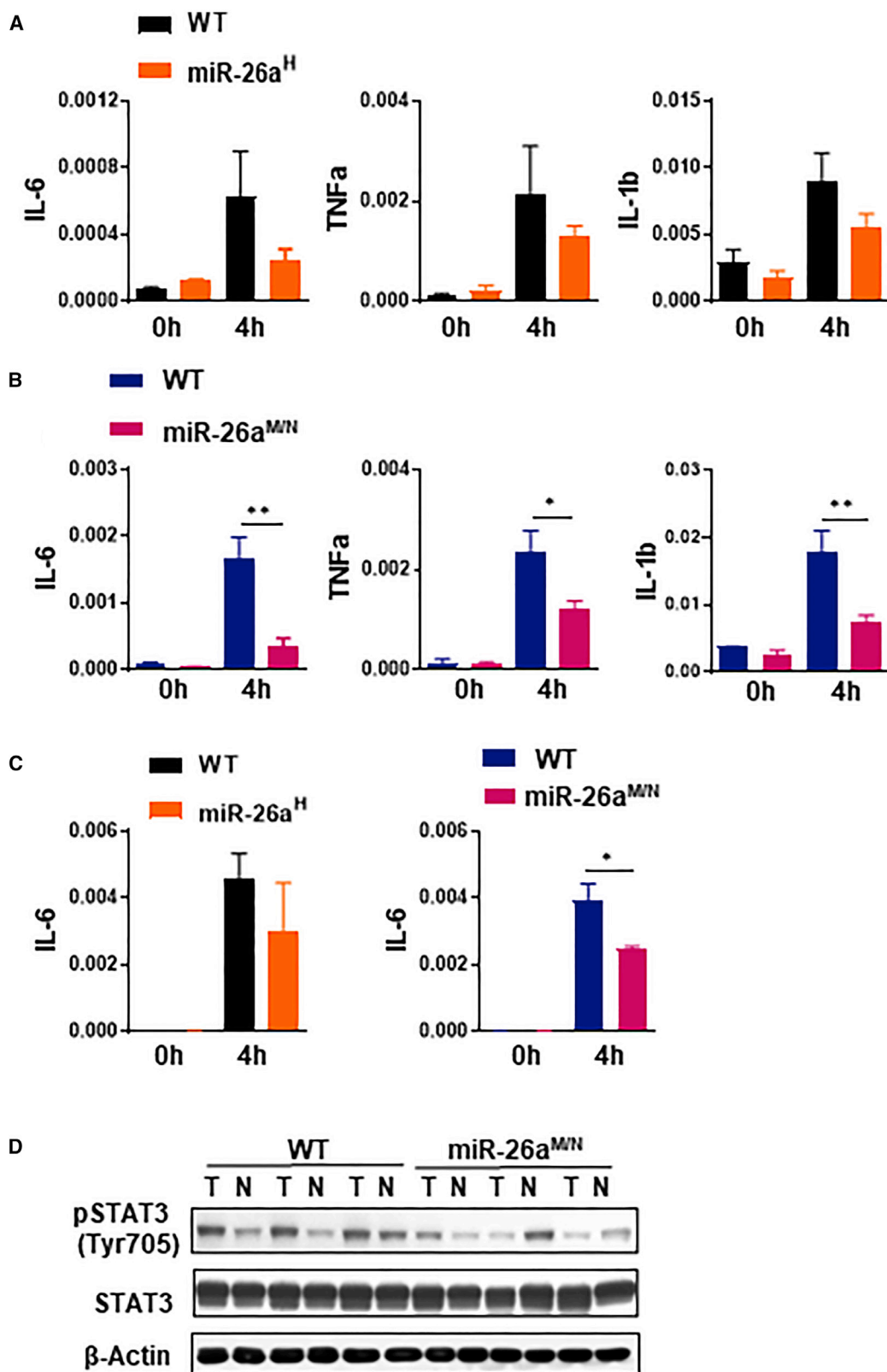
We previously showed that miR-26a significantly reduced the luciferase activity of the IL-6 reporter containing a WT 3' untranslated region (UTR) but not a mutant 3'-UTR (Figure S6), suggesting that IL-6 is a direct target of miR-26a.³¹ In addition to IL-6, we ask whether

there are other miR-26a targets that are potentially involved in the regulation of macrophage activity, including TLR3,³² PKC δ ,³³ GSK3 β ,³⁴ and PTEN.^{35,36} To answer this question,

HEK293T cells were transfected with reporter constructs containing the 3' UTR of selected target genes and co-transfected with either miR-26a mimics (miR-26a) or NCs. These dual-luciferase reporter assays showed that coexpression of miR-26a, but not NCs, significantly suppressed the activity of firefly luciferase carrying the 3' UTR of TLR3, PTEN, GSK3 β , PKC δ , and ZFP462 (Figure 7A). In contrast, miR-26a inhibitor increased the expression of the same genes. We selected two genes (TLR3-1 and PKC δ) to mutate the putative miR-26a-binding sites. The results showed that miR-26a failed to activate the reporter genes containing the mutants, suggesting that at least TLR3-1 and PKC δ were bona fide miR-26a direct target genes (Figure 7B). The effect of miR-26a on the endogenous cellular expression of the above-mentioned targets was examined by quantitative reverse transcriptase PCR in RAW264.7 and J774A.1 cells. The results showed that introduction of miR-26a significantly decreased the expression of the mRNA levels of TLR3, PKC δ , GSK3 β , PTEN, and RTF1 (Figures 7C and S7A), which were further confirmed by the reduced protein levels of PKC δ , GSK3 β , and PTEN (Figures 7D and S7B). Taken together, the results indicated that miR-26a may directly inhibit a number of key genes that are involved in regulating liver inflammation.

DISCUSSION

HCC is characteristically associated with the pathologically chronic liver diseases hepatitis and cirrhosis. For example, in the DEN-induced HCC model, which causes chronic liver injury, hepatocytes proliferate continuously and at higher rates than normal liver cells. This uncontrolled proliferation or irregular liver regeneration is usually triggered by several cytokines in the liver, including TNF- α and IL-6.^{37–43} Moreover, the critical roles of IL-6 and Kupffer cells in HCC development are further confirmed by its link to the gender disparity in HCC and obesity-enhanced HCC development.^{44,45} Therefore, understanding the concerted regulatory functions of different liver cells will provide mechanistic insights into the pathogenesis of HCC as well as help develop potential approaches to target several liver cell types simultaneously for HCC treatment.



(legend on next page)

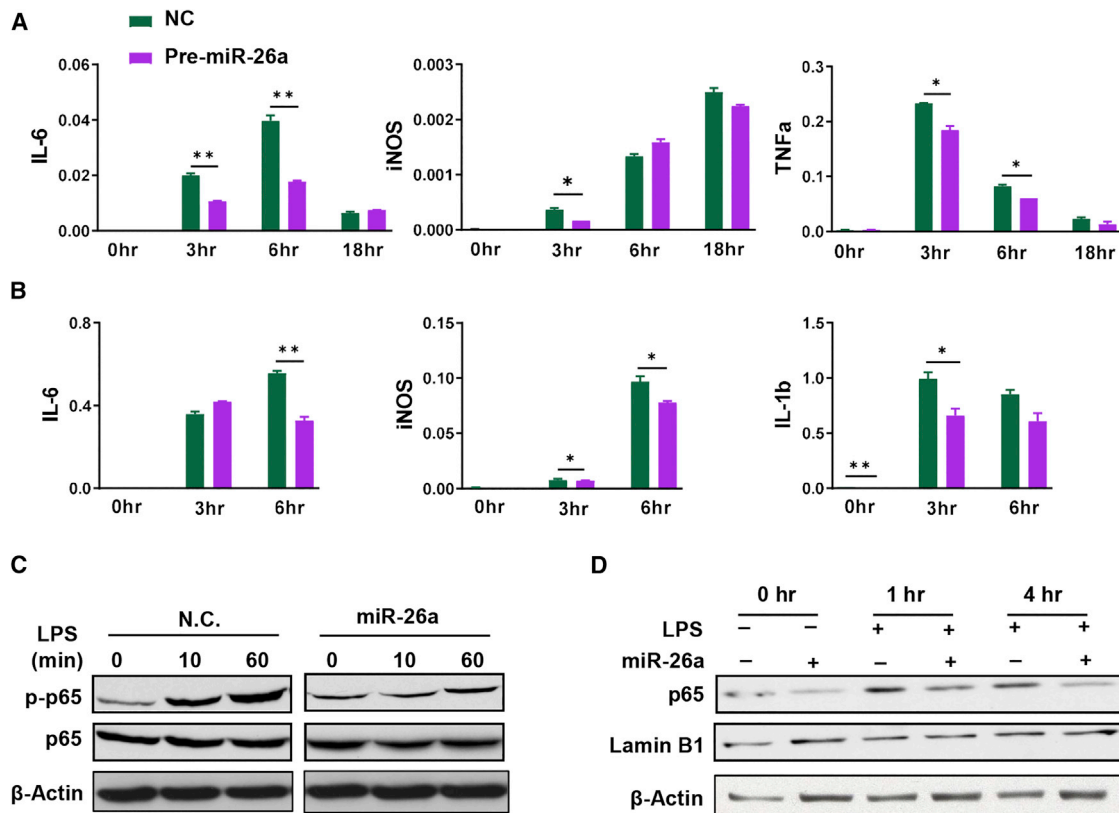


Figure 6. miR-26a overexpression in macrophages reduces LPS-induced inflammation *in vitro*

(A) qRT-PCR analysis of expression of genes promoting inflammation in RAW264.7 cells transfected with an miR-26a mimic (miR-26a) or negative control (NC), followed by LPS (10 ng/mL) treatment for different time points as indicated. mRNA expression levels were normalized to those of β -actin. * $p < 0.05$; ** $p < 0.01$. (B) qRT-PCR analysis of expression of genes promoting inflammation in bone marrow-derived macrophages from miR-26a^{M/N} and their WT littermates treated with LPS (10 ng/mL) for different time points as indicated. mRNA expression levels were normalized to those of β -actin. * $p < 0.05$; ** $p < 0.01$. (C) Representative western blot analysis of p65 in RAW264.7 cells transfected with an miR-26a mimic (miR-26a) or NC, followed by LPS (10 ng/mL) treatment for different time points as indicated. (D) Representative western blot analysis of p65 in RAW264.7 cells transfected with an miR-26a mimic (+) or NC (–), followed by LPS (10 ng/mL) treatment for different time points as indicated.

miRNAs are emerging new therapeutic targets. In the case of miR-26a, there is strong clinical evidence for its potent tumor suppressing roles in HCC.^{20,21} Although therapeutic modulation of miRNAs is still an emerging field, the ability to inhibit groups of genes that regulate cancer growth and to target tumor cells as well as the tumor microenvironment (which would be possible by targeting miR-26a) has tremendous potential for cancer treatment. Previous studies also identified miR-26a as a potential tumor suppressor in HCC and other cancers.^{23,25,46} In the present study, we used cell-specific miR-26Tg mice to demonstrate a potent effect of miR-26a in attenuating DEN-induced HCC. We demonstrate that cell-specific overexpression of miR-26 in either hepatocytes or myeloid cells is sufficient to inhibit HCC development in mice. Because hepatocytes and

Kupffer cells are well-established major cell types during hepatocarcinogenesis, our findings not only confirm the previous observations but also provide a potential miRNA-mediated therapeutic approach to treat HCC. Therefore, miR-26a mimics may have the advantage in targeting both liver cancer cells and Kupffer cells to achieve better therapeutic outcomes compared to most of the drugs that target cancer cells only. Furthermore, it would be interesting to investigate whether miR-26a also has functions in other liver cancer-infiltrated immune cells, such as T cells and B cells. A few reports do support a potential role of miR-26a in cancer-associated T cells,^{47,48} which suggest a suppressive role of miR-26a in CD8⁺ cytotoxic T lymphocyte responses in the tumor microenvironment. It would be interesting to investigate whether this role of miR-26a can be observed

Figure 5. miR-26a overexpression in myeloid cells reduces chemical-induced acute inflammation *in vivo*

(A and B) qRT-PCR analysis of expression of genes promoting inflammation in the livers of DEN-treated miR-26a^H (A), miR-26a^{M/N} (B), and their WT littermate mice. (C) qRT-PCR analysis of IL-6 expression in the liver of LPS-treated miR-26a^{M/N} and WT littermate controls. (D) Representative western blot analysis of STAT3 in DEN-induced liver tumors from miR-26a^{M/N} and WT littermate control mice. mRNA expression levels were normalized to those of β -actin. * $p < 0.05$; ** $p < 0.01$.

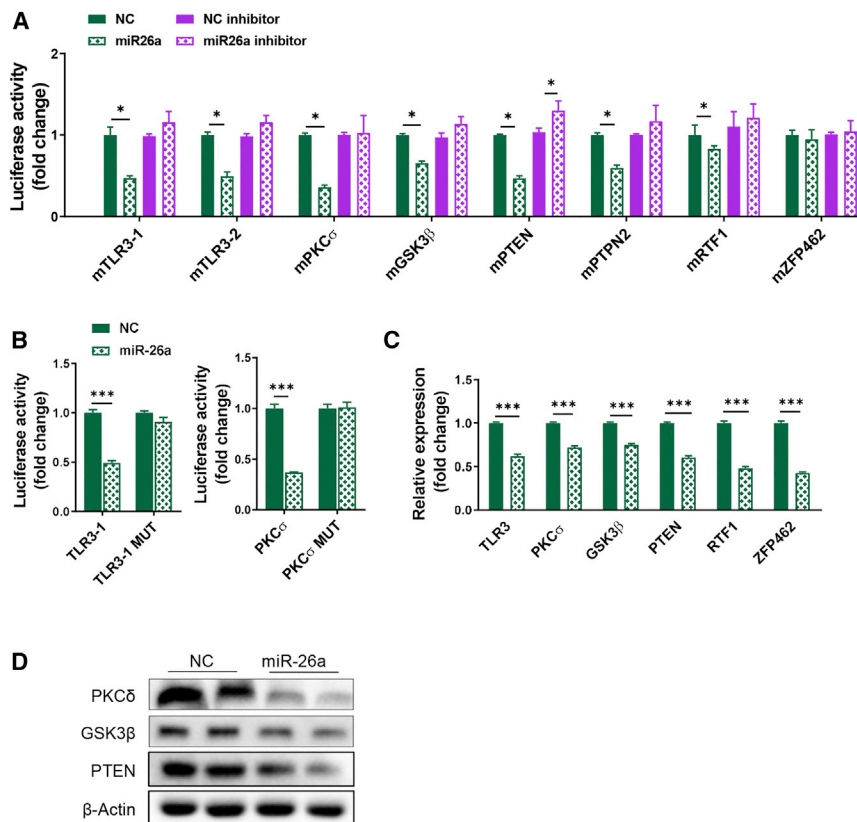


Figure 7. miR-26a targets a number of genes in regulating liver inflammation

(A) Relative luciferase activity in HEK293T cells transfected with reporter constructs containing the 3' UTR of target genes and co-transfected with either miR-26a mimics (miR-26a) or NC. (B) Expression of miR-26a target genes in RAW264.7 cells transfected with an miR-26a mimic (miR-26a) or NC. Mut, mutant. (C) qRT-PCR analysis of genes in RAW264.7 cells transfected with an miR-26a mimic (miR-26a) or NC. mRNA expression levels were normalized to those of β -actin. * $p < 0.05$; *** $p < 0.005$. (D) Raw264.7 cells were transfected with miR-26a mimic (miR-26a) for 24 h, and cell lysates were prepared and subjected to analysis by western blotting using the antibodies as indicated.

inflammation, such as chronic viral hepatitis or nonalcoholic steatohepatitis, because inflammation also plays a critical role in promoting these types of hepatitis and they have been shown to be closely linked to hepatocarcinogenesis.

In summary, overexpression of miR-26a in either hepatocytes or Kupffer cells confer mice resistant to DEN-induced hepatocarcinogenesis. The strong suppression of either NF- κ B or STAT3 signaling pathways in hepatocytes and Kupffer cells may underlie the miR-26a-mediated HCC attenuation. These findings thus

provide mechanistic insights into a concerted role of miR-26a in both hepatocytes and Kupffer cells to suppress hepatocarcinogenesis, which highlight the potential use of miR-26a mimetics as therapeutic approaches for the treatment of HCC.

MATERIALS AND METHODS

Animal models

We have generated miR-26a transgenic (TG) mice as shown in [Figure S1](#) and as described in a previous study.¹⁴ Hypoxanthine guanine phosphoribosyltransferase-Cre mice were bred with mice carrying the target allele to delete the Neo-STOP cassette during embryogenesis.⁴⁹ The Albumin-Cre and LysM-Cre mice (from The Jackson Laboratory) were bred with mice carrying the target allele to selectively delete the Neo-STOP cassette in hepatocytes or macrophages.⁵⁰ Heterozygous transgenic mice and their littermate WT mice were used for experiments. All of the procedures followed the guidelines for the care and use of laboratory animals from the National Institutes of Health and Institutional Animal Care and Use Committees (IACUCs) of individual institutions.

Induction of HCC

Three DEN-induced HCC models were conducted following a protocol as described previously.²³ In the first model, 6- to 8-week-old mice were injected intraperitoneally with DEN (100 mg/kg body weight, Sigma). After 7 days, 3 mg/kg TCPOBOP was injected

in the HCC tumor microenvironment, which would be our future endeavor.

Although a variety of miR-26a target genes have been identified, we and others have consistently shown that suppression of inflammatory pathways during carcinogenesis is key to the tumor-suppressor function of miR-26a. We have recently shown that miR-26a attenuates colitis and colitis-associated cancer by downregulating multiple intestinal inflammatory pathways.³¹ Similarly, in this study, both *in vivo* and *in vitro* data suggest that miR-26a strongly reduces hepatic inflammation during DEN-induced hepatocarcinogenesis. Both acute DEN and LPS treatments in mice result in lower expression levels of pro-inflammatory cytokines in miR-26a transgenic mice. Transfection of miR-26a in either two macrophage cell lines or BMDMs resulted in lower levels of TNF- α and IL-6 when stimulated by LPS. Furthermore, miR-26a repressed the LPS-induced phosphorylation of p65 and its nuclear translocation, indicating a role of miR-26a in suppressing this cytokine production by negatively regulating NF- κ B signaling in macrophages. More interestingly, we have provided evidence that IL-6 could be a direct miR-26a target gene. miR-26a overexpression also led to reduced STAT3 phosphorylation. Taken together, these results indicated that NF- κ B and STAT3 signaling pathways may be two major targets of miR-26a during hepatocarcinogenesis. We anticipate that miR-26a mimics may be also effective in suppressing some chronic liver diseases related to chronic

intraperitoneally every 2 weeks for 6 times, and mice were euthanized 4–5 months after the DEN injection. In the second model, 15-day-old mice were injected intraperitoneally with DEN (25 mg/kg body weight), followed by 4 instances of TCPOBOP (3 mg/kg body weight) intraperitoneal injection every 2 weeks, and mice were euthanized 4–5 months after the DEN injection. In the third model, 15-day-old mice were injected intraperitoneally with DEN (25 mg/kg body weight), and mice were euthanized 8 months after the DEN injection.

Induction of acute liver inflammation

Mice were injected intraperitoneally with DEN (100 mg/kg body weight) or LPS (20 mg/kg body weight) to induce acute inflammation. Mice were euthanized and livers were collected for gene expression analysis at 4 h post-DEN injection or 6 h post-LPS injection.

Cell culture and transfection

HEK293T and RAW264.7 cells were purchased from the American Type Culture Collection. J774A.1 cells were provided by Dr. Mingye Feng at City of Hope. Transfection of plasmids was performed with Attractene (Qiagen, Valencia, CA) according to the manufacturer's instructions. The miRNA mimic was purchased from Ambion (Austin, TX) and Exiqon (Vedbaek, Denmark), respectively. Transfections of miRNAs or inhibitors were performed using HiPerfect (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Real-Time-PCR

Real-time-PCR was performed using the Power SYBR Green PCR Master Mix protocol (Applied Biosystems, Foster City, CA). To analyze mRNAs, reverse transcription was performed with Superscript III reverse transcriptase and Oligo(dT) 20 primers (Invitrogen, Carlsbad, CA) at 50°C for 1 h. The expression levels of miRNAs were normalized to those of 5S RNA. mRNA expression levels were normalized to those of β -actin (Ambion, Austin, TX). The sequences of the primers used are listed in Table S1.

ELISA

The protein levels of TNF- α and IL-6 in mouse livers and cell cultures were detected by using specific ELISA kits according to the manufacturer's guidelines. ELISA kits were purchased from Proteintech Group (TNF- α , KE10002; IL-6, KE10007). Protein concentrations of liver and cell lysates were used to normalize the cytokine levels.

Western blotting

Both liver tissues and cell lysates were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis before transferring to nitrocellulose membranes. After blocking in 5% nonfat milk, the membranes were incubated with the following primary antibodies: anti-phospho-STAT3, anti-total STAT3, anti-I κ B α , anti-PKC δ , and anti-PTEN obtained from Cell Signaling Technology (Danvers, MA); anti-GSK3 β from Abcam (Cambridge, UK); and anti-p38, anti-pERK, and anti-GAPDH from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes were washed and incubated with peroxidase-conjugated secondary antibodies (Amersham Bioscience, Amersham, UK).

BMDM culture and stimulation

BM cells were harvested from the femurs of mice and filtered through a 70- μ m strainer. After red blood cells were lysed with ACK lysis buffer (Thermo Fisher, Waltham, MA), the cell pellet was washed and suspended in DMEM containing 20% FBS and 10 ng/mL murine M-CSF (BioLegend, San Diego, CA), and cells were plated in a 6-well tissue culture plate at 2×10^6 cells per well and incubated for 7 days. To activate BMDMs, 10 ng/mL LPS (Sigma) was added to the medium. Six hours later, cells were harvested for analysis.

Luciferase activity assays

HEK293T cells were transfected with 40 nM miRNA precursors (GenePharma, Shanghai, China) and 200 ng psiCHECK-2.2 (Promega, Madison, WI) constructs containing an insert of the 3' UTR or flanking sequences of seed nucleotides of miR-26a target genes using Attractene (Qiagen, Valencia, CA) in 96-well plates. Twenty-four hours after transfection, cells were analyzed with a dual-luciferase reporter assay (Promega). To generate mutant reporter constructs, the seed sequence of miR-26a binding site was mutated using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA).

Statistical analysis

Student's *t* test (two-sided) was applied, and changes were considered statistically significant if $p < 0.05$. In the figures, changes are noted using * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.005$. The data shown in the bar graphs are the means and SDs of at least three biological replicates. The statistical analysis was conducted using Microsoft Excel or GraphPad Prism software.

DATA AND CODE AVAILABILITY

The authors declare that all data supporting the findings of this study are available within the paper and its [supplemental information files](#).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2022.08.036>.

ACKNOWLEDGMENTS

We thank Dr. Ian Talisman from City of Hope (COH) for his help in manuscript proofreading. We thank the funding support from the National Cancer Institute (2R01CA139158, to W.H. and H.Y.), the National Institute of Diabetes and Digestive and Kidney Diseases (R01DK124627, to W.H.), the National Natural Science Foundation of China (82172986, to Y.T.; 81970561 and 92157205, to X.F.). The research reported in this publication also included work performed in the following Cores: Pathology Research Services, Light Microscopy/Digital Imaging Core, which are supported by the National Cancer Institute of the NIH under award number P30CA33572, as well as the Animal Resource Center and the Transgenic/Knockout Animal Cores at COH.

AUTHOR CONTRIBUTIONS

W.H. and X.F. conceived and designed the study; Y.T., M.Z., M.F., H.X., S.W., W.H., S.Z., Y.W., D.T., C.Z., and X.F. performed the

experiments; W.H., X.F., Y.T., M.Z., and M.F. helped analyze the data and prepared the figures; W.H., X.F., and H.Y. wrote and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Llovet, J.M., Burroughs, A., and Bruix, J. (2003). Hepatocellular carcinoma. *Lancet* 362, 1907–1917. [https://doi.org/10.1016/S0140-6736\(03\)14964-1](https://doi.org/10.1016/S0140-6736(03)14964-1).
- Shen, C., Zhao, C.Y., Zhang, R., and Qiao, L. (2012). Obesity-related hepatocellular carcinoma: roles of risk factors altered in obesity. *Front. Biosci.* 17, 2356–2370.
- Lian, Z., Liu, J., Wu, M., Wang, H.Y., Arbuthnot, P., Kew, M., and Feitelson, M.A. (2007). Hepatitis B x antigen up-regulates vascular endothelial growth factor receptor 3 in hepatocarcinogenesis. *Hepatology* 45, 1390–1399. <https://doi.org/10.1002/hep.21610>.
- Whittaker, S., Marais, R., and Zhu, A.X. (2010). The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene* 29, 4989–5005. <https://doi.org/10.1038/onc.2010.236>.
- Hahn, O., and Stadler, W. (2006). Sorafenib. *Curr. Opin. Oncol.* 18, 615–621. <https://doi.org/10.1097/01.cco.0000245316.82391.52>.
- Llovet, J.M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.F., de Oliveira, A.C., Santoro, A., Raoul, J.L., Forner, A., et al. (2008). Sorafenib in advanced hepatocellular carcinoma. *N. Engl. J. Med.* 359, 378–390. <https://doi.org/10.1056/NEJMoa0708857>.
- Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297. [https://doi.org/10.1016/S0092-8674\(04\)00045-5](https://doi.org/10.1016/S0092-8674(04)00045-5).
- Negrini, M., Nicoloso, M.S., and Calin, G.A. (2009). MicroRNAs and cancer—new paradigms in molecular oncology. *Curr. Opin. Cell Biol.* 21, 470–479. <https://doi.org/10.1016/j.ccb.2009.03.002>.
- Lujambio, A., and Lowe, S.W. (2012). The microcosmos of cancer. *Nature* 482, 347–355. <https://doi.org/10.1038/nature10888>.
- Li, Z., and Rana, T.M. (2014). Therapeutic targeting of microRNAs: current status and future challenges. *Nat. Rev. Drug Discov.* 13, 622–638. <https://doi.org/10.1038/nrd4359>.
- van Rooij, E., and Kauppinen, S. (2014). Development of microRNA therapeutics is coming of age. *EMBO Mol. Med.* 6, 851–864. <https://doi.org/10.1525/emmm.201100899>.
- Callegari, E., Gramantieri, L., Domenicali, M., D'Abundo, L., Sabbioni, S., and Negrini, M. (2015). MicroRNAs in liver cancer: a model for investigating pathogenesis and novel therapeutic approaches. *Cell Death Differ.* 22, 46–57. <https://doi.org/10.1038/cdd.2014.136>.
- Lin, S., and Gregory, R.I. (2015). MicroRNA biogenesis pathways in cancer. *Nat. Rev. Cancer* 15, 321–333. <https://doi.org/10.1038/nrc3932>.
- Fu, X., Jin, L., Wang, X., Luo, A., Hu, J., Zheng, X., Tsark, W.M., Riggs, A.D., Ku, H.T., and Huang, W. (2013). MicroRNA-26a targets ten eleven translocation enzymes and is regulated during pancreatic cell differentiation. *Proc. Natl. Acad. Sci. USA* 110, 17892–17897. <https://doi.org/10.1073/pnas.1317397110>.
- Xu, H., Tian, Y., Tang, D., Zou, S., Liu, G., Song, J., Zhang, G., Du, X., Huang, W., He, B., et al. (2021). An endoplasmic reticulum stress-MicroRNA-26a feedback circuit in NAFLD. *Hepatology* 73, 1327–1345. <https://doi.org/10.1002/hep.31428>.
- Han, W., Fu, X., Xie, J., Meng, Z., Gu, Y., Wang, X., Li, L., Pan, H., and Huang, W. (2015). MiR-26a enhances autophagy to protect against ethanol-induced acute liver injury. *J. Mol. Med. (Berl.)* 93, 1045–1055. <https://doi.org/10.1007/s00109-015-1282-2>.
- Fu, X., Dong, B., Tian, Y., Lefebvre, P., Meng, Z., Wang, X., Pattou, F., Han, W., Wang, X., Lou, F., et al. (2015). MicroRNA-26a regulates insulin sensitivity and metabolism of glucose and lipids. *J. Clin. Invest.* 125, 2497–2509. <https://doi.org/10.1172/JCI75438>.
- Liu, G., Du, W., Xu, H., Sun, Q., Tang, D., Zou, S., Zhang, Y., Ma, M., Zhang, G., Du, X., et al. (2020). RNA G-quadruplex regulates microRNA-26a biogenesis and function. *J. Hepatol.* 73, 371–382. <https://doi.org/10.1016/j.jhep.2020.02.032>.
- Fu, X., Meng, Z., Liang, W., Tian, Y., Wang, X., Han, W., Lou, G., Wang, X., Lou, F., Yen, Y., et al. (2014). miR-26a enhances miRNA biogenesis by targeting Lin28B and Zcchc11 to suppress tumor growth and metastasis. *Oncogene* 33, 4296–4306. <https://doi.org/10.1038/onc.2013.385>.
- Ji, J., Shi, J., Budhu, A., Yu, Z., Forgues, M., Roessler, S., Ambis, S., Chen, Y., Meltzer, P.S., Croce, C.M., et al. (2009). MicroRNA expression, survival, and response to interferon in liver cancer. *N. Engl. J. Med.* 361, 1437–1447. <https://doi.org/10.1056/NEJMoa0901282>.
- Yang, X., Liang, L., Zhang, X.F., Jia, H.L., Qin, Y., Zhu, X.C., Gao, X.M., Qiao, P., Zheng, Y., Sheng, Y.Y., et al. (2013). MicroRNA-26a suppresses tumor growth and metastasis of human hepatocellular carcinoma by targeting interleukin-6-Stat3 pathway. *Hepatology* 58, 158–170. <https://doi.org/10.1002/hep.26305>.
- Meng, Z., Fu, X., Chen, X., Zeng, S., Tian, Y., Jove, R., Xu, R., and Huang, W. (2010). miR-194 is a marker of hepatic epithelial cells and suppresses metastasis of liver cancer cells in mice. *Hepatology* 52, 2148–2157. <https://doi.org/10.1002/hep.23915>.
- Yang, F., Huang, X., Yi, T., Yen, Y., Moore, D.D., and Huang, W. (2007). Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. *Cancer Res.* 67, 863–867. <https://doi.org/10.1158/0008-5472.Can-06-1078>.
- Liu, N., Meng, Z., Lou, G., Zhou, W., Wang, X., Zhang, Y., Zhang, L., Liu, X., Yen, Y., Lai, L., et al. (2012). Hepatocarcinogenesis in FXR-/- mice mimics human HCC progression that operates through HNF1alpha regulation of FXR expression. *Mol. Endocrinol.* 26, 775–785. <https://doi.org/10.1210/me.2011-1383>.
- Kota, J., Chivukula, R.R., O'Donnell, K.A., Wentzel, E.A., Montgomery, C.L., Hwang, H.W., Chang, T.C., Vivekanandan, P., Torbenson, M., Clark, K.R., et al. (2009). Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* 137, 1005–1017. <https://doi.org/10.1016/j.cell.2009.04.021>.
- Huang, W., Ma, K., Zhang, J., Qatanani, M., Cuvillier, J., Liu, J., Dong, B., Huang, X., and Moore, D.D. (2006). Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. *Science* 312, 233–236. <https://doi.org/10.1126/science.1121435>.
- Huang, W., Zhang, J., Washington, M., Liu, J., Parant, J.M., Lozano, G., and Moore, D.D. (2005). Xenobiotic stress induces hepatomegaly and liver tumors via the nuclear receptor constitutive androstane receptor. *Mol. Endocrinol.* 19, 1646–1653. <https://doi.org/10.1210/me.2004-0520>.
- Sahu, S.K., Kumar, M., Chakraborty, S., Banerjee, S.K., Kumar, R., Gupta, P., Jana, K., Gupta, U.D., Ghosh, Z., Kundu, M., and Basu, J. (2017). MicroRNA 26a (miR-26a)/KLF4 and CREB-C/EBPbeta regulate innate immune signaling, the polarization of macrophages and the trafficking of Mycobacterium tuberculosis to lysosomes during infection. *PLoS Pathog.* 13, e1006410. <https://doi.org/10.1371/journal.ppat.1006410>.
- Chai, Z.T., Zhu, X.D., Ao, J.Y., Wang, W.Q., Gao, D.M., Kong, J., Zhang, N., Zhang, Y.Y., Ye, B.G., Ma, D.N., et al. (2015). microRNA-26a suppresses recruitment of macrophages by down-regulating macrophage colony-stimulating factor expression through the PI3K/Akt pathway in hepatocellular carcinoma. *J. Hematol. Oncol.* 8, 56. <https://doi.org/10.1186/s13045-015-0150-4>.
- Ni, B., Rajaram, M.V., Lafuse, W.P., Landes, M.B., and Schlesinger, L.S. (2014). Mycobacterium tuberculosis decreases human macrophage IFN-gamma responsiveness through miR-132 and miR-26a. *J. Immunol.* 193, 4537–4547. <https://doi.org/10.4049/jimmunol.1400124>.
- Zhang, W., Fu, X., Xie, J., Pan, H., Han, W., and Huang, W. (2021). miR-26a attenuates colitis and colitis-associated cancer by targeting the multiple intestinal inflammatory pathways. *Mol. Ther. Nucleic Acids* 24, 264–273. <https://doi.org/10.1016/j.omtn.2021.02.029>.
- Tatematsu, M., Seya, T., and Matsumoto, M. (2014). Beyond dsRNA: toll-like receptor 3 signalling in RNA-induced immune responses. *Biochem. J.* 458, 195–201. <https://doi.org/10.1042/BJ20131492>.
- Loefering, D.J., and Lennartz, M.R. (2011). Protein kinase C and toll-like receptor signaling. *Enzyme Res.* 537821. <https://doi.org/10.4061/2011/537821>.
- Wang, F.S., Fan, J.G., Zhang, Z., Gao, B., and Wang, H.Y. (2014). The global burden of liver disease: the major impact of China. *Hepatology* 60, 2099–2108. <https://doi.org/10.1002/hep.27406>.

35. Schabbauer, G., Matt, U., Gunzl, P., Warszawska, J., Furtner, T., Hainzl, E., Elbau, I., Mesteri, I., Doninger, B., Binder, B.R., and Knapp, S. (2010). Myeloid PTEN promotes inflammation but impairs bactericidal activities during murine pneumococcal pneumonia. *J. Immunol.* 185, 468–476. <https://doi.org/10.4049/jimmunol.0902221>.
36. Sahin, E., Haubenwallner, S., Kuttke, M., Kollmann, I., Halfmann, A., Dohnal, A.M., Chen, L., Cheng, P., Hoesel, B., Einwallner, E., et al. (2014). Macrophage PTEN regulates expression and secretion of arginase I modulating innate and adaptive immune responses. *J. Immunol.* 193, 1717–1727. <https://doi.org/10.4049/jimmunol.1302167>.
37. Coussens, F.B.a.L.M. (2004). An Inflammatory Link.
38. Pikarsky, E., Porat, R.M., Stein, I., Abramovitch, R., Amit, S., Kasem, S., Gutkovich-Pyest, E., Urieli-Shoval, S., Galun, E., and Ben-Neriah, Y. (2004). NF- κ B Functions as a Tumour Promoter in Inflammation-Associated Cancer.
39. Maeda, S., Kamata, H., Luo, J.L., Leffert, H., and Karin, M. (2005). IKK β couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 121, 977–990. <https://doi.org/10.1016/j.cell.2005.04.014>.
40. Toshiharu Sakurai, S.M., Lufen C and Michael K Loss of Hepatic NF- κ B Activity Enhances Chemical Hepatocarcinogenesis through Sustained C-Jun N-Terminal Kinase 1 Activation.
41. Luedde, T., Beraza, N., Kotsikoris, V., van Loo, G., Nenci, A., De Vos, R., Roskams, T., Trautwein, C., and Pasparakis, M. (2007). Deletion of NEMO/IKK γ in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell* 11, 119–132. <https://doi.org/10.1016/j.ccr.2006.12.016>.
42. Shibata, M., Morizane, T., Uchida, T., Yamagami, T., Onozuka, Y., Nakano, M., Mitamura, K., and Ueno, Y. (1998). Irregular regeneration of hepatocytes and risk of hepatocellular carcinoma in chronic hepatitis and cirrhosis with hepatitis-C-virus infection. *Lancet* 351, 1773–1777. [https://doi.org/10.1016/s0140-6736\(97\)08002-1](https://doi.org/10.1016/s0140-6736(97)08002-1).
43. Ueno, Y., Moriyama, M., Uchida, T., and Arakawa, Y. (2001). Irregular regeneration of hepatocytes is an important factor in the hepatocarcinogenesis of liver disease. *Hepatology* 33, 357–362. <https://doi.org/10.1053/jhep.2001.21902>.
44. Naugler WE, S.T., Kim, S., Maeda, S., Kim, K., Elsharkawy, A.M., and Karin, M. (2007). Gender Disparity in Liver Cancer Due to Sex Differences in MyD88-dependent IL-6 Production.
45. Park, E.J., Lee, J.H., Yu, G.Y., He, G., Ali, S.R., Holzer, R.G., Osterreicher, C.H., Takahashi, H., and Karin, M. (2010). Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell* 140, 197–208. <https://doi.org/10.1016/j.cell.2009.12.052>.
46. Yang, X., Zhang, X.F., Lu, X., Jia, H.L., Liang, L., Dong, Q.Z., Ye, Q.H., and Qin, L.X. (2014). MicroRNA-26a suppresses angiogenesis in human hepatocellular carcinoma by targeting hepatocyte growth factor-cMet pathway. *Hepatology* 59, 1874–1885. <https://doi.org/10.1002/hep.26941>.
47. Long, H., Xiang, T., Luo, J., Li, F., Lin, R., Liu, S., Jiang, S., Hu, C., Chen, G., Wong, E., et al. (2016). The tumor microenvironment disarms CD8(+) T lymphocyte function via a miR-26a-EZH2 axis. *Oncoimmunology* 5, e1245267. <https://doi.org/10.1080/2162402X.2016.1245267>.
48. Zhao, E., Maj, T., Kryczek, I., Li, W., Wu, K., Zhao, L., Wei, S., Crespo, J., Wan, S., Vatan, L., et al. (2016). Cancer mediates effector T cell dysfunction by targeting microRNAs and EZH2 via glycolysis restriction. *Nat. Immunol.* 17, 95–103. <https://doi.org/10.1038/ni.3313>.
49. Tang, S.H., Silva, F.J., Tsark, W.M., and Mann, J.R. (2002). A Cre/loxP-deleter transgenic line in mouse strain 129S1/SvImJ. *Genesis* 32, 199–202. <https://doi.org/10.1002/gene.10030>.
50. Clausen, B.E., Burkhardt, C., Reith, W., and Förster, R.R. (1999). Conditional Gene Targeting in Macrophages and Granulocytes Using LysMcre Mice.