Published in final edited form as:

Hepatology. 2016 March ; 63(3): 864–879. doi:10.1002/hep.28367.

Differentiation Therapy for Hepatocellular Carcinoma: multifaceted effects of miR-148a on tumor growth and phenotype and liver fibrosis

Kwang Hwa Jung^{1,*}, Jing Zhang^{1,*}, Chong Zhou¹, Hong Shen¹, Mihai Gagea², Cristian Rodriguez-Aguayo³, Gabriel Lopez-Berestein³, Anil K. Sood⁴, and Laura Beretta¹

¹Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

²Department of Veterinary Medicine & Surgery, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

³Department of Experimental Therapeutics and the Center for RNAi and Non-Coding RNA, The University of Texas MD Anderson Cancer Center, Houston, TX 77054, USA

⁴Department of Gynecologic and Reproductive Oncology and the Center for RNAi and Non-Coding RNA, The University of Texas MD Anderson Cancer Center, Houston, TX 77054, USA

Abstract

Death rate from HCC is increasing and liver cancer is the second leading cause of cancer-related mortality worldwide. Most patients with HCC have underlying liver cirrhosis and compromised liver function, limiting treatment options. Cirrhosis is associated with cell dedifferentiation and expansion of hepatocholangiolar progenitor cells. We identified a miRNA signature associated with HCC and hepatocytic differentiation of progenitor cells. We further identified miR-148a as an inducer of hepatocytic differentiation that is downregulated in HCC. MiR-148a-mimetic treatment in vivo suppressed tumor growth, reduced tumor malignancy and liver fibrosis, and prevented tumor development. These effects were associated with an increased differentiated phenotype and were mediated by IKK α /NUMB/NOTCH signaling.

Conclusion—our results identified miR-148a as an inhibitor of the IKK α /NUMB/NOTCH pathway and an inducer of hepatocytic differentiation that when deregulated promotes HCC initiation and progression. This study represents the first evidence that differentiation-targeted therapy is a promising strategy to treat and prevent HCC.

Keywords

HCC; MiR-148a; Differentiation therapy; IKK α /NUMB/NOTCH signaling

Correspondence to Laura Beretta, Ph.D., Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA. lberetta@mdanderson.org phone: (713) 792-9100, Fax: (713) 794-4023.

*These authors contributed equally to this work.

Conflict of Interest: The authors declare no conflict of interest.

Introduction

Hepatocellular Carcinoma (HCC) is the second most common cause of cancer-related deaths in the world.^{1,2} Although the highest liver cancer rates are found in certain areas of Asia and Africa, liver cancer incidence and mortality rates are increasing in western countries, including the United States.³ Progression of HCC is characterized by abnormal cell differentiation, fast infiltrating growth, early metastasis, high-grade malignancy, and poor prognosis.^{4,5} Liver cirrhosis due to hepatitis B virus (HBV), hepatitis C virus (HCV), high alcohol consumption or non-alcoholic steatohepatitis (NASH) are the main risk factors associated with HCC. Most patients with HCC have underlying liver cirrhosis and compromised liver function, limiting treatment options. Long-term survival rates are low and novel approaches to prevent and treat HCC are urgently needed.⁶

MicroRNAs (miRNAs) are noncoding RNAs 18–25 nucleotides in length that regulate a variety of biological processes by silencing their cognate target genes.⁷ There is ample evidence that miRNAs have regulatory functions in human cancer initiation and progression and may act as oncogenes or tumor suppressor.^{8–10} Deregulation of miRNAs occurs frequently in a variety of liver diseases, including HCC.^{11,12} Aberrant miRNA expression is associated with clinical features of HCC, such as occurrence, development and stage.¹³ Increasing amounts of evidence have shown that miRNAs play important roles in regulating liver development and homeostasis as well. For example, miR-30a regulates vertebrate bile duct development¹⁴ and miR-23b cluster miRNAs are required for repression of bile duct gene expression in fetal hepatocytes.¹⁵ MiR-122, a liver specific miRNA, is required for proper progression of hepatocyte differentiation^{16,17} and has been implicated in several hepatic disorders.^{18,19}

Together, these findings suggest that deregulation of miRNA could contribute to the aberrant regulation of differentiation associated genes, and confers oncogenic potential to cancer cells during hepatocarcinogenesis. In addition, it is now accepted that the arrested differentiation of tissue-based stem cells or their progenitor cells is linked to hepatocarcinogenesis.²⁰ Therefore, the fact that miRNAs have dual regulating effects in cell differentiation and liver cancer development led us to hypothesize that microRNAs that modulate liver progenitor cell fate could be targeted for differentiation therapy. Whereby, the increased numbers of liver cancer stem cells or liver progenitor cells present in cirrhotic liver are induced to differentiate, and in doing so lose their self-renewal capacity and reduces their tumorigenic potential. We used mice with hepatocyte-specific deletion of *Pten* to test this hypothesis as these mice develop progressive disease with presence of steatosis and fibrosis characteristic of NASH preceding the development of HCC.^{21–23} Accumulation of liver progenitor cells preceding tumor development and poorly differentiated phenotype of the tumors have also been described in this model, making it highly suitable for the proposed study.

Materials and Methods

Detailed materials and methods used in this study are given in the Supporting Information.

Cell Culture and Hepatocytic Differentiation of HepaRG cells

Human hepatoma cell line Huh7 was grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin. HepaRG liver progenitor cells were cultured in William's E medium (Invitrogen) supplemented with 10% FBS (Sigma), 100 units/mL penicillin, 100 µg/mL streptomycin (Invitrogen), 5 µg/mL insulin (Sigma) and 50 µM hydrocortisone hemisuccinate (Sigma). A two-step procedure was used to induce hepatocytic differentiation of HepaRG cells as previously described.^{24,25} Briefly, HepaRG cells (1.5×10^5 cells) were cultured in complete medium for two weeks. Then, the culture medium was supplemented with 1% DMSO (Sigma) and 20 ng/mL epidermal growth factor (EGF; Peprotech) for two additional weeks. The medium was renewed every 2 or 3 days. Cells were harvested at 2, 14, and 28 days after seeding, and pictures were taken using a phase contrast microscope (Nikon).

Mice Treatment

Mouse studies were approved by the MDACC Institutional Animal Care and Use Committee. C57BL/6 mice carrying *Pten* conditional knockout alleles were crossed with an Albumin (Alb)-Cre-transgenic mouse. For this model, control animals are *Pten*^{loxP/loxP}; Alb-Cre⁻ while the experimental mice are *Pten*^{loxP/loxP}; Alb-Cre⁺. For *in vivo* miR-148a delivery, nanoliposomal miRNA was prepared as previously described.²⁶ Briefly, miR-148a was incorporated into nanoliposomes made from 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) in presence of excess t-butanol. After Tween 20 addition, mixture was then frozen, lyophilized, and stored at -80°C. Before *in vivo* administration, the preparation was rehydrated with PBS to achieve desired dose per injection. Hepatic *Pten* null mice (7.5 month-old or 10.5 month-old) were injected intraperitoneally with single dose of miR-148a/DOPC liposomes (final concentration of 5 µg per 200 µL). Treatment (5 µg miRNA per injection) continued for 6 weeks with 2 injections per week at 3 to 4 day intervals (total 12 injections per mouse). For *in vivo* Notch inhibition, hepatic *Pten* null mice (8 month-old or 11 month-old) received RO4929097 (10 mg/kg, Selleckchem) in 1% Klucel in water with 0.2% Tween 80 daily by oral gavage for 4 weeks. Each treatment group included 8–12 mice.

Quantitative PCR

For quantitation of mature miRNAs, reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit in a reaction mixture containing a miR-specific stem-loop reverse transcription (RT) primer. The quantification of mature miRNAs was performed with TaqMan primers in a universal PCR master mix in ViiA7 Real-Time PCR System (Applied Biosystems). To quantify target gene expression levels, equal amounts of RNA samples were submitted to reverse transcription and real-time PCR using specific primers listed in Supporting Table 1. PCR amplifications of the respective genes were performed with iTaq SYBR Green Supermix (Bio-Rad) in CFX Connect Real-Time System (Bio-Rad). The Bio-Rad CFX Manager software (version 2.1) was used for calculation of threshold cycles (Ct)-values and melting curve analysis of amplified DNA. Relative expression of the tested miRNAs and genes was calculated by 2^{-Ct} method.

Results

MiRNA Signature Associated with Hepatocytic Differentiation and HCC

We wanted to identify microRNAs that are regulated during hepatocytic differentiation of liver progenitor cells and inversely regulated in HCC. To that end, we performed miRNA expression profiling analysis in HepaRG liver progenitor cells at the proliferative (day 2) and differentiated (day 28) stages. In addition, miRNA expression profiling analysis was performed in Huh7 hepatoma cells and healthy human liver. We identified seven miRNAs that were changed upon hepatocytic differentiation of HepaRG cells and inversely regulated in Huh7 cells compared to healthy liver (Fig. 1A). Expression of miR-148a, miR-150, miR-101 and miR-29c were upregulated upon hepatocytic differentiation of HepaRG cells while downregulated in Huh7 cells compared to healthy liver. In contrast, expression of miR-93, miR-221 and miR-18a was downregulated upon hepatocytic differentiation of HepaRG cells while upregulated in Huh7 cells. The deregulation of these 7 miRNAs in HCC was further confirmed using 5 public datasets available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession numbers: GSE22058, GSE10694, GSE21362., GSE39678 and GSE36915) (Supporting Table 2). The expression of the 7 miRNAs in the largest HCC dataset (accession number: GSE22058) is shown in Figure 1B. Expression of miR-148a, miR-150, miR-101 and miR-29c was significantly downregulated while expression of miR-93, miR-221 and miR-18a was significantly upregulated in HCC compared to adjacent liver tissue (Fig. 1B).

MiR-148a Promotes Hepatocytic Differentiation of HepaRG Liver Progenitor and HCC Cells, and its Expression Correlates with Hepatocytic Markers in HCC

To determine whether these seven miRNAs (miR-148a, miR-150, miR-101, miR-29c, miR-93, miR-221 and miR-18a) have the capacity to regulate hepatocytic differentiation, we established HepaRG cell lines overexpressing individually these miRNAs. Overexpression of each miRNA compared to control empty vector cell lines, was confirmed by qRT-PCR analysis (Fig. 2A). Whereas no morphologic differences were noticed between the different cell lines at the proliferative stage, striking morphological differences were observed at day 14 of the differentiation process in miR-148a overexpressing HepaRG cell lines and in miR-101 overexpressing HepaRG cell lines compared to empty control cell lines (Fig. 2B). Indeed, even before addition of differentiation medium, a significantly greater density of bile canaliculi was observed at day 14 in miR-148a overexpressing cells (13,871 canaliculi/cm²) and in miR-101 overexpressing cells (12,056 canaliculi/cm²) compared to empty control cells (5,031 canaliculi/cm²) (Fig. 2C, left panel). Similar results were observed at day 28 (Fig. 2C, right panel). Expression of the hepatocyte markers HNF4A, ALDOB, ALB and CYP3A4 and of the hepatocyte-specific microRNA miR-122, was also significantly higher in miR-148a overexpressing HepaRG cells compared to empty control cells (4.06-fold, p=0.004; 2.11-fold, p=0.031; 2.27-fold, p=0.028; 1.46-fold, p=0.043 and 9.45-fold, p=0.047, respectively) (Fig. 2D). We further validated by qRT-PCR that miR-148a expression increases in HepaRG cells upon hepatocytic differentiation and that this finding can be generalized to human embryonic stem cells upon directed hepatocytic differentiation (Supporting Fig. 1).

To further evaluate whether downregulation of miR-148a in HCC is associated with hepatocytic differentiation status of human tumors, we performed a correlation analysis between miR-148a and the hepatocytic markers HNF4A, ALDOB, CYP3A4 and miR-122, using the same large NCBI GEO HCC dataset (accession number: GSE22058). MiR-148a levels strongly correlated with all four hepatocytic markers with R values ranging from 0.482–0.574 ($p<0.001$) (Supporting Table 3). These results were further validated for HNF4A, CYP3A4 and miR-122 in a separate dataset (accession number: GSE20596) (Supporting Table 3). We finally overexpressed miR-148a in the HCC cell lines, PLC/PRF/5 and Huh7. Enforced expression of miR-148a in PLC/PRF/5 cells resulted in a significant increase in the expression of the hepatocyte markers HNF4A and ALB, concomitant to a significant decrease in AFP expression (1.77-fold, $p=0.026$; 2.41-fold, $p=0.019$; –1.58-fold, $p=0.027$, respectively). Similar results were obtained in the Huh7 cells (1.53-fold, $p=0.028$; 1.67-fold, $p=0.037$; –1.28-fold, $p=0.018$, for HNF4A, ALB and AFP respectively) (Supporting Fig. 2).

Altogether, these results demonstrate that miR-148a promotes hepatocytic differentiation of progenitor cells and HCC cells and that its reduced expression in HCC is associated with reduced differentiation status of the tumors. MiR-148a is therefore a relevant target for differentiation therapy in HCC.

MiR-148a-mimetic Treatment Reduces Tumor Growth, Tumor Malignancy and Liver Fibrosis in vivo

Before performing any *in vivo* study, it was important to identify miR-148a targets that could be used to validate target delivery and activity. To that end, we used the program miRWalk (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>) to select genes predicted to be targets of miR-148a by at least two prediction algorithms. We then integrated this gene list with a list of genes downregulated upon hepatocytic differentiation of HepaRG cells that we previously reported.²⁵ From these integrated analyses, we identified 97 genes that are predicted to be target of miR-148a and that are downregulated upon hepatocytic differentiation of HepaRG cells (Supporting Table 4). Of these 97 genes, we selected for validation a known miR-148a target, DNMT1, and two genes known for their role in cell differentiation, CENPF and MYBL1. All three genes were significantly downregulated upon miR-148a overexpression in HepaRG cells confirming that they can be used to measure the efficiency of target delivery *in vivo* (Supporting Fig. 3A).

We treated 10.5 month-old male mice with hepatic deletion of *Pten* that had developed tumors confirmed by using Magnetic Resonance Imaging (MRI), with miR-148a packaged into DOPC liposomes (5 μ g per mouse) or with placebo. After 6 weeks of treatment with bi-weekly intraperitoneal injections, tumors and adjacent liver tissues were collected and processed for gene expression and histology analysis. MiR-148a treatment efficiency was validated by measuring miR-148a target genes DNMT1, CENPF and MYBL1. Upon miR-148a treatment, expressions of DNMT1, CENPF and MYBL1 were significantly decreased (–1.64-fold, $p<0.001$; –1.36-fold, $p=0.02$ and –1.48-fold, $p=0.045$, respectively) (Supporting Fig. 3B). MiR-148a treatment efficiency was also validated by measuring miR-148a in liver and tumors of miR-148a-treated mice (Supporting Fig. 3C). We next

evaluated the effect of miR-148a treatment on tumor growth. MRI showed increased tumor size in the placebo treated group during the length of the treatment while tumor size in the miR-148a treated group remained largely unchanged (Fig. 3A). This observation was confirmed by MRI data analysis. The average tumor growth rate from over 14 days was 2.04-fold in the placebo group compared to 1.14-fold in the miR-148a treated group ($p<0.001$) (Fig. 3B). Inhibition of tumor growth by miR-148a treatment was further confirmed by tumor size distribution. While 65.71% of the tumors collected from the placebo group were $>50 \text{ mm}^3$, only 37.76% of the tumors in miR-148a treated mice were $>50 \text{ mm}^3$ ($p=0.049$) (Fig. 3C). Blinded histology analysis of the tumors by a pathologist showed that while in the placebo group, the majority of the tumors were HCC (69.2%), all collected tumors in the miR-148a treated group were hepatocellular and cholangiocellular adenomas, demonstrating that miR-148a treatment resulted not only in decreased tumor growth but also in reduced malignancy (Fig. 3D). Since *Pten* null mice develop in addition to HCC, liver fibrosis and steatosis, we also evaluated the effect of miR-148a treatment on these underlying pathologies. While miR-148a treatment didn't significantly affect the degree of steatosis (Supporting Fig. 4A), it significantly reduced fibrosis from 32.2% to 22.8% ($p<0.001$) (Fig. 3E).

MiR-148a-mimetic Treatment Induces a Hepatic Differentiation Phenotype in vivo

In order to evaluate whether the effects of miR-148a treatment *in vivo* were associated with increased hepatic differentiation, we measured the expression of progenitor cell, biliary cell and hepatocyte cell markers. Expression of progenitor cell markers CD24 and osteopontin (OPN) was significantly reduced in miR-148a treated tumors compared to placebo tumors (-2.01 -fold, $p=0.02$ and -4.24 -fold, $p=0.049$, respectively) (Fig. 4A). Expression of biliary cell markers KRT19 and SOX9 was also significantly reduced after miR-148a treatment (-2.36 -fold, $p=0.013$ and -3.37 -fold, $p<0.001$, respectively) (Fig. 4B). In contrast, expression of hepatocyte markers HNF4 α and miR-122 was significantly increased in miR-148a treated tumors (1.52-fold, $p=0.035$ and 1.34-fold, $p=0.021$, respectively) (Fig. 4C). We further performed dual-label immunofluorescence experiments using two progenitor cell specific antibodies: A6 (progenitor cell and newly formed hepatocyte markers) and EPCAM (progenitor cell marker) in liver tissues. Immunofluorescence analysis revealed an accumulation of A6+/EPCAM- cells in miR-148a treated liver whereas almost no accumulation of A6+/EPCAM- cells was observed in the placebo group ($p<0.001$) (Fig. 4D). Taken together, these data suggest that miR-148a promotes hepatocytic differentiation in tumors and adjacent liver.

MiR-148a Treatment Prevents Tumor Development, Reduces liver fibrosis and Improves Liver function in vivo

We then evaluated whether miR-148a could prevent tumor development in *Pten* null mice. We treated 7.5 month-old male mice with hepatic deletion of *Pten*, before they had developed tumors as confirmed using MRI, with miR-148a/DOPC or with placebo for 6 weeks. At the end of treatment, tumor incidence rate decreased from 75% in the placebo group to 33.33% in miR-148a treated group (Fig. 5A). Significant decrease in tumor volume average and in tumor burden was also observed in miR-148a treated mice compared to placebo group (5.17 mm^3 vs 44.11 mm^3 , $p=0.046$ and 6.67 mm^3 vs 87.84 mm^3 , $p=0.008$,

respectively) (Fig. 5B). Blinded histological analysis by a pathologist showed that while the majority of the tumors that developed in the placebo group were HCC (57.1%), all tumors detected in the miR-148a treated group were hepatocellular and cholangiocellular adenomas (Fig. 5C). As observed in the first set of *Pten* null treated mice, fibrosis was significantly reduced from 6.31% to 3.77% ($p=0.044$) upon miR-148a treatment while steatosis was unchanged (Fig. 5D). Finally, serum biochemical analysis showed a significant improvement in liver function upon miR-148a treatment as AST levels decreased from 298 IU/L to 166 IU/L in miR-148a treated group ($p=0.05$) (Fig. 5E).

IKK α – NOTCH signaling are regulated by MiR-148a and mediates miR-148a-induced hepatocytic differentiation

To determine the mechanisms of miR-148a mediated tumor effects, we used the list of 97 genes that are predicted miR-148a targets and are downregulated upon HepaRG hepatocytic differentiation. We focused on I κ B kinase alpha (IKK α) because of its role in HCC tumorigenesis.²⁷ Conserved miR-148a target sites are present in the 3'UTR of human and mouse IKK α (Fig. 6A, left panel) and downregulation of IKK α expression during hepatocytic differentiation of HepaRG cells was confirmed by PCR (-1.52 -fold, $p=0.025$) (Fig. 6A, middle panel). Expression of IKK α mRNA and protein was decreased in miR-148a overexpressing HepaRG cell lines (Supporting Fig. 5A). This effect was further validated by transient transfection of miR-148a in HepaRG cell lines (Supporting Fig. 5B). To confirm that miR-148a directly targets IKK α , we co-transfected HepaRG cells with miR-148a and a luciferase expression vector containing the 3'UTR of IKK α . Overexpression of miR-148a significantly decreased luciferase activity of the reporter gene containing the IKK α 3'UTR (Fig. 6A, right panel). We further showed that IKK α expression was suppressed in tumors of miR-148a treated mice compared to tumors of placebo group ($p=0.019$) (Supporting Fig. 6A).

Because it has been reported that IKK α mediates NOTCH activation through regulation of NUMB in HCC,²⁷ we investigated the effect of miR-148a on IKK α /NUMB/NOTCH signaling pathway. Our results showed that NUMB, negative regulator of NOTCH signaling, was significantly upregulated in tumors of miR-148a treated mice compared to tumors of placebo group ($p=0.023$), whereas NOTCH downstream genes HES1 and HEY1 expression levels were suppressed (-1.46 -fold, $p=0.018$ and -1.85 -fold, $p<0.001$, respectively) (Supporting Fig. 6B), suggesting that miR-148a inactivates the IKK α /NUMB/NOTCH pathway in tumors of *Pten* null mice. In human HCCs, the expression of IKK α is significantly upregulated, NUMB expression is significantly downregulated and HEY1 expression is significantly upregulated (Supporting Fig. 6C). Taken together, these results indicate that miR-148a is novel repressor of IKK α /NUMB/NOTCH signaling pathway by directly targeting IKK α expression.

We further evaluated whether IKK α and NOTCH mediate the miR-148a-induced hepatocytic differentiation. To that end, we established stable IKK α knockdown HepaRG cell lines, and confirmed the silencing of IKK α expression in established cell lines (HepaRG-IKK α KD) (Fig. 6B, left panel). A significantly greater density of bile canaliculi was observed in HepaRG-IKK α KD cells compared to empty control cells upon hepatocytic

differentiation (7,025 vs 4,138 canaliculi/cm² at day 14, $p < 0.001$ and 11,341 vs 8,156 canaliculi/cm² at day 28, $p = 0.003$) (Fig. 6B, middle panel). Expression of HNF4A, ALDOB, ALB and miR-122 was also significantly increased at day 14 in HepaRG- $IKK\alpha$ KD cells compared to empty control cells (1.85-fold, $p = 0.047$; 1.66-fold, $p = 0.037$; 2.80-fold, $p = 0.012$ and 2.35-fold, $p = 0.038$, respectively) (Fig. 6B, right panel). We then investigated whether overexpression of NOTCH could reverse the miR-148a-induced hepatocytic differentiation. We selected to overexpress NOTCH2 as we previously reported that NOTCH2 is the most abundant NOTCH family member in liver progenitor cells.⁴² MiR-148a-overexpressing HepaRG cell lines stably overexpressing NOTCH2 were generated (HepaRG-miR-148a-NOTCH2) and NOTCH2 overexpression was confirmed by qRT-PCR (Fig. 6C, left panel). NOTCH2 overexpression didn't affect the levels of miR-148a in these cell lines (Fig. 6C, left panel). At days 14 and 28 of the hepatocytic differentiation process, NOTCH2 overexpression reverted the increase in bile canaliculi density observed in HepaRG-miR-148a cells (Fig. 6C, middle panel). NOTCH2 overexpression also reverted the increased expression of HNF4A, ALDOB, ALB and miR-122 observed in HepaRG-miR-148a cells at day 14 (Fig. 6C, right panel). Altogether, these results show that $IKK\alpha$ silencing has a similar effect on hepatocytic differentiation as compared to miR-148a overexpression and that NOTCH2 overexpression can revert miR-148a-induced hepatocytic differentiation *in vitro*, demonstrating that $IKK\alpha$ -NOTCH pathway mediates miR-148a effect on hepatocytic differentiation.

NOTCH Inhibitor (RO4929097) Treatment Reduces Tumor Growth, Tumor Malignancy and Liver Fibrosis in vivo, effects associated with Increased Hepatic Differentiation Phenotype

To further investigate whether NOTCH mediates miR-148a-induced tumor suppression, we treated 11 month-old male *Pten* null mice that had developed tumors as confirmed by MRI, with RO4929097, an inhibitor of Notch activity. After 4 weeks of treatment with daily injections, expression of HEY1 significantly decreased (−1.6-fold; $p = 0.013$), confirming that RO4929097 treatment was effective in blocking NOTCH activity (Fig. 7A). We next evaluated the effect of RO4929097 on tumor growth. RO4929097 treatment significantly decreased tumor growth from 2.04-fold over 14 days in the placebo group to 1.48-fold in the RO4929097 treated group ($p < 0.001$) (Fig. 7B). Inhibition of tumor growth following RO4929097 treatment was further demonstrated by the tumor size distribution per mouse in both groups. While 61.2% of the tumors in placebo-treated mice were $> 50 \text{ mm}^3$, only 30.4% of the tumors in RO4929097 treated mice were $> 50 \text{ mm}^3$ ($p = 0.047$) (Fig. 7C). Similar to the effect of miR-148a treatment on tumor malignancy, the incidence of hepatocellular carcinoma and hepatocholangiocellular carcinoma was decreased from 76.9% in the placebo group to 9.1% in the RO4929097 treated group as most tumors detected in the RO4929097 treated group were hepatocellular and cholangiocellular adenomas (90.9%) (Fig. 7D). Also similar to miR-148a treatment, liver fibrosis decreased from 33.1% to 18.9% ($p = 0.009$) upon RO4929097 treatment (Fig. 7E) while no significant effect on steatosis was observed (Supporting Fig. 4B). Following RO4929097 treatment, expression of liver progenitor cell markers CD24 and OPN was significantly reduced (−1.93-fold, $p = 0.008$ and −1.78-fold, $p = 0.044$, respectively) (Supporting Fig. 7A). Expression of biliary cell markers KRT19 and SOX9 was also significantly reduced (−3.98-fold, $p = 0.006$ and −1.7-fold, $p = 0.019$, respectively) (Supporting Fig. 7B). In contrast, expression of hepatocyte marker miR-122

was significantly increased (1.3-fold, $p=0.021$) (Supporting Fig. 7C). Co-staining for EPCAM and A6 showed no accumulation of A6+/EPCAM- cells in liver tissues of placebo treated group, while RO4929097 treatment induced an accumulation of A6+/EPCAM- cells ($p=0.011$) (Supporting Fig. 7D). Altogether, these results show similar effects of Notch inhibition on differentiation phenotype, as observed for miR-148a treatment.

NOTCH inhibitor (RO4929097) Treatment Prevents Tumor Development in vivo

Lastly, we investigated whether inhibition of NOTCH activity could prevent tumor development in *Pten* null mice. Following treatment of RO4929097 for 4 weeks, tumor incidence was reduced from 75% in the placebo group to 33.3% in the RO4929097 treated group (Fig. 8A). The average tumor volume and tumor burden in RO4929097 treated mice were significantly lower than in the placebo group (7.03 mm^3 vs 46.3 mm^3 , $p=0.03$ and 15.05 mm^3 vs 84.51 mm^3 , $p=0.028$, respectively) (Fig. 8B). Additionally, the incidence of hepatocellular carcinoma was decreased from 57.1% in the placebo group to 20% in the RO4929097 treated group (Fig. 8C). Liver fibrosis was also significantly reduced from 4.57% to 2.18% ($p=0.031$) upon RO4929097 treatment (Fig. 8D).

Discussion

HCC has the fastest growing death rate among cancers in the United States, at a time that witnesses a decrease in cancer mortality. Survival has not significantly improved in decades and the 5-year survival rate remains under 15%. The large majority of HCC cases are diagnosed at a late stage and a challenge in the clinical care of HCC patients is that over 90% of patients with HCC have underlying liver cirrhosis and compromised liver function, limiting treatment options. Liver cirrhosis of any etiology is the main risk factor associated with HCC and eventually progresses into HCC in 3–5% of patients per year.²⁸ Treatment of HCC remains a huge unmet need. Over the past few years, cell differentiation-driven strategies to treat solid tumors have emerged based on insights into the biology of tumors as well as on identification of pathways capable of controlling cell fate programs that are deregulated in these tumors. The new paradigm of cellular differentiation, once viewed as a unidirectional process, is now recognized as a plastic process in which cells can dedifferentiate into cells with stem-like phenotypes. Because plastic phenotypic shifts are likely to influence the early steps of HCC initiation in cirrhosis, the use of differentiation-targeted therapy should be considered for HCC treatment and chemoprevention. Whether the tumor initiating cells originate from hepatic stem/progenitor cells or dedifferentiated hepatocytes, targeting the regulatory mechanism of their self-renewal and differentiation capacity is a promising strategy for both therapy and chemoprevention.

MiRNAs have been shown to play essential roles in cell fate and cancer development.^{29,30} In this study, we identified a miRNA signature that is associated with HCC and inversely associated with hepatocytic differentiation. Among these miRNAs, we showed that miR-148a is a potent inducer of hepatocytic differentiation. We further showed that miR-148a is downregulated in HCC and levels of miR-148a in HCC strongly correlates with the differentiation phenotype of the tumors as measured by expression levels of hepatocytic markers. The downregulation of miR-148a in HCC appears to be independent of the

etiology (Supporting Fig. 8A) and miR-148a expression is not affected by the presence or not of cirrhosis in the tumor adjacent liver (Supporting Fig. 8B).

A role for miR-148a in promoting terminal cell differentiation was previously reported for myoblasts, B cells and hepatoblasts.^{31–33} The later study reported that miR-148a induces hepatocytic differentiation of mouse fetal hepatoblasts.³² These results together with ours suggest that the role of miR-148a as an inducer of hepatocytic differentiation, is conserved in human and mouse. Expression of miR-148a has been shown to be downregulated in various cancers and associated with poor prognosis.^{34,35} In HCC, lower levels of miR-148a are associated with poor prognosis and define a cancer stem cell-like aggressive subtype.^{36,37} *In vitro* studies showed that enforced expression of miR-148a suppresses the motility and invasive abilities of HCC cells and growth of the invasive MHCC97H cells in xenograft model, is reduced upon miR-148a treatment.^{32,37} Together, these prior studies suggested that miR-148a could have utility for the treatment of aggressive and invasive HCCs. Our study is the first to evaluate the effect of miR-148a treatment *in vivo* in a genetically engineered mouse model of HCC and to demonstrate that miR-148a treatment reduces growth of non-invasive tumors, an effect we demonstrate to be associated with *in vivo* hepatocytic differentiation and phenotypic change of the tumor. Our study is also the first to evaluate miR-148a as a chemoprevention target. In addition, we are reporting for the first time a beneficial effect of miR-148a treatment on liver fibrosis and liver function.

We identified IKK α as a direct target of miR-148a. The nuclear factor (NF)- κ B pathway is tightly regulated by the IKK complex, which consists of two catalytic subunits, IKK α and IKK β , and the NF- κ B essential modulator NEMO.³⁸ Several studies have demonstrated the tumor promoter role of IKK α in human cancers including liver cancer.^{27,39–41} Moreover, elevated expression of IKK α in HCC has been associated with activation of the NUMB/NOTCH pathway.²⁷ We further demonstrated that indeed, by targeting IKK α , miR-148a inhibits NUMB/NOTCH signaling pathway *in vivo*. We further showed that IKK α is downregulated upon hepatocytic differentiation of liver progenitor HepaRG cells. We also recently reported that NOTCH2 is highly expressed in liver progenitor cells and necessary for the survival of CD24+ liver progenitor cells.⁴² Therefore, in liver cells, IKK α seems to act as a negative regulator of hepatocytic differentiation and a promoter of stem cell maintenance. Similar results were found in prostate with IKK α required for expansion of epithelial progenitors.³⁹ In contrast, IKK α promotes epidermal differentiation and prevents skin cancer.⁴³

The inhibitory effect of miR-148a on NOTCH activity is particularly relevant to hepatocarcinogenesis and the role of cell plasticity in this process. NOTCH signaling is highly evolutionarily conserved and is involved in cell fate control during development, stem cell self-renewal and cell differentiation.⁴⁴ The role of NOTCH signaling is particularly important in liver biology and pathology.^{45,46} Activated NOTCH signaling induces hepatic tumors in mice.^{47–50} These studies are in agreement with our study demonstrating that inhibition of NOTCH signaling by RO4929097 treatment effectively decreased tumor growth and prevented tumor development, suggesting that it may serve as a therapeutic target for liver cancer.

In conclusion, this study identified miR-148a as a highly promising target for the therapeutic treatment and prevention of HCC. Alternative targets include IKK α and NOTCH. Not only miR-148a treatment inhibits tumor growth and prevents tumor development, miR-148a treatment also reduces the malignancy of the hepatic tumors and the amount of fibrosis in affected livers, improving liver function. Such effects are particularly well suited for the treatment of patients with HCC and underlying liver cirrhosis. Finally, this study is a proof-of-concept that differentiation-targeted therapy is a promising approach for liver cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: This work was supported in part by the MD Anderson Cancer Center Support Grant CA016672, through a Multidisciplinary Research Program Award, by the the RGK Foundation and U54 CA151668.

We thank Donjeta Gjuka, Dr. Jingjing Jiao (Department of Molecular and Cellular Oncology, University of Texas MD Anderson Cancer Center), Dr. Ryan L. McCarthy and Dr. Michelle C. Barton (Department of Epigenetics and Molecular Carcinogenesis, University of Texas MD Anderson Cancer Center) for their help in experimental procedure.

Abbreviations

ALDOB	fructose-bisphosphate aldolase B
CYP3A4	cytochrome P450 3A4
DOPC	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HNF4A	hepatocyte nuclear factor 4 alpha
IKKα	I κ B kinase alpha
MRI	magnetic resonance imaging
miRNA	microRNA
mRNA	messenger RNA
NASH	nonalcoholic steatohepatitis
OPN	osteopontin
qRT-PCR	quantitative real-time polymerase chain reaction

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011; 61:69–90. [PubMed: 21296855]

2. Kim HY, Park JW. Clinical trials of combined molecular targeted therapy and locoregional therapy in hepatocellular carcinoma: past, present, and future. *Liver Cancer*. 2014; 3:9–17. [PubMed: 24804173]
3. El-Serag HB. Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology*. 2004; 127:S27–34. [PubMed: 15508094]
4. Lazarevich NL, Cheremnova OA, Varga EV, Ovchinnikov DA, Kudrjavitseva EI, Morozova OV, et al. Progression of HCC in mice is associated with a downregulation in the expression of hepatocyte nuclear factors. *Hepatology*. 2004; 39:1038–1047. [PubMed: 15057908]
5. Tanaka S, Arai S. Molecular targeted therapies in hepatocellular carcinoma. *Semin Oncol*. 2012; 39:486–492. [PubMed: 22846865]
6. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology*. 2011; 53:1020–1022. [PubMed: 21374666]
7. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004; 116:281–297. [PubMed: 14744438]
8. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 2006; 6:857–866. [PubMed: 17060945]
9. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*. 2006; 6:259–269. [PubMed: 16557279]
10. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005; 435:834–838. [PubMed: 15944708]
11. Szabo G, Bala S. MicroRNAs in liver disease. *Nat Rev Gastroenterol Hepatol*. 2013; 10:542–552. [PubMed: 23689081]
12. Wang XW, Heegaard NH, Orum H. MicroRNAs in liver disease. *Gastroenterology*. 2012; 142:1431–1443. [PubMed: 22504185]
13. Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, Shimotohno K. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene*. 2006; 25:2537–2545. [PubMed: 16331254]
14. Hand NJ, Master ZR, Eaclaure SF, Weinblatt DE, Matthews RP, Friedman JR. The microRNA-30 family is required for vertebrate hepatobiliary development. *Gastroenterology*. 2009; 136:1081–1090. [PubMed: 19185580]
15. Rogler CE, Levoci L, Ader T, Massimi A, Tchaikovskaya T, Norel R, Rogler LE. MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads. *Hepatology*. 2009; 50:575–584. [PubMed: 19582816]
16. Doddapaneni R, Chawla YK, Das A, Kalra JK, Ghosh S, Chakraborti A. Overexpression of microRNA-122 enhances in vitro hepatic differentiation of fetal liver-derived stem/progenitor cells. *J Cell Biochem*. 2013; 114:1575–1583. [PubMed: 23334867]
17. Laudadio I, Manfroid I, Achouri Y, Schmidt D, Wilson MD, Cordi S, et al. A feedback loop between the liver-enriched transcription factor network and miR-122 controls hepatocyte differentiation. *Gastroenterology*. 2012; 142:119–129. [PubMed: 21920465]
18. Starkey Lewis PJ, Dear J, Platt V, Simpson KJ, Craig DG, Antoine DJ, et al. Circulating microRNAs as potential markers of human drug-induced liver injury. *Hepatology*. 2011; 54:1767–1776. [PubMed: 22045675]
19. Trebicka J, Anadol E, Elfimova N, Strack I, Roggendorf M, Viazov S, et al. Hepatic and serum levels of miR-122 after chronic HCV-induced fibrosis. *J Hepatol*. 2013; 58:234–239. [PubMed: 23085648]
20. Kung JW, Currie IS, Forbes SJ, Ross JA. Liver development, regeneration, and carcinogenesis. *J Biomed Biotechnol*. 2010; 2010:984248. [PubMed: 20169172]
21. Horie Y, Suzuki A, Kataoka E, Sasaki T, Hamada K, Sasaki J, et al. Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. *J Clin Invest*. 2004; 113:1774–1783. [PubMed: 15199412]
22. Stiles B, Wang Y, Stahl A, Bassilian S, Lee WP, Kim YJ, et al. Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity [corrected]. *Proc Natl Acad Sci U S A*. 2004; 101:2082–2087. [PubMed: 14769918]

23. Muir K, Hazim A, He Y, Peyressatre M, Kim DY, Song X, Beretta L. Proteomic and lipidomic signatures of lipid metabolism in NASH-associated hepatocellular carcinoma. *Cancer Res.* 2013; 73:4722–4731. [PubMed: 23749645]
24. Parent R, Marion MJ, Furio L, Trepo C, Petit MA. Origin and characterization of a human bipotent liver progenitor cell line. *Gastroenterology.* 2004; 126:1147–1156. [PubMed: 15057753]
25. Parent R, Beretta L. Translational control plays a prominent role in the hepatocytic differentiation of HepaRG liver progenitor cells. *Genome Biol.* 2008; 9:R19. [PubMed: 18221535]
26. Landen CN Jr, Chavez-Reyes A, Bucana C, Schmandt R, Deavers MT, Lopez-Berestein G, Sood AK. Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. *Cancer Res.* 2005; 65:6910–6918. [PubMed: 16061675]
27. Liu M, Lee DF, Chen CT, Yen CJ, Li LY, Lee HJ, et al. IKKalpha activation of NOTCH links tumorigenesis via FOXA2 suppression. *Mol Cell.* 2012; 45:171–184. [PubMed: 22196886]
28. Michelotti GA, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. *Nat Rev Gastroenterol Hepatol.* 2013; 10:656–665. [PubMed: 24080776]
29. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. *Nat Rev Drug Discov.* 2010; 9:775–789. [PubMed: 20885409]
30. Ivey KN, Srivastava D. MicroRNAs as regulators of differentiation and cell fate decisions. *Cell Stem Cell.* 2010; 7:36–41. [PubMed: 20621048]
31. Zhang J, Ying ZZ, Tang ZL, Long LQ, Li K. MicroRNA-148a promotes myogenic differentiation by targeting the ROCK1 gene. *J Biol Chem.* 2012; 287:21093–21101. [PubMed: 22547064]
32. Gailhouse L, Gomez-Santos L, Hagiwara K, Hatada I, Kitagawa N, Kawaharada K, et al. miR-148a plays a pivotal role in the liver by promoting the hepatospecific phenotype and suppressing the invasiveness of transformed cells. *Hepatology.* 2013; 58:1153–1165. [PubMed: 23532995]
33. Porstner M, Winkelmann R, Daum P, Schmid J, Pracht K, Corte-Real J, et al. miR-148a promotes plasma cell differentiation and targets the germinal center transcription factors Mitf and Bach2. *Eur J Immunol.* 2015; 45:1206–1215. [PubMed: 25678371]
34. Zheng B, Liang L, Wang C, Huang S, Cao X, Zha R, et al. MicroRNA-148a suppresses tumor cell invasion and metastasis by downregulating ROCK1 in gastric cancer. *Clin Cancer Res.* 2011; 17:7574–7583. [PubMed: 21994419]
35. Kim J, Zhang Y, Skalski M, Hayes J, Kefas B, Schiff D, et al. microRNA-148a is a prognostic oncomiR that targets MIG6 and BIM to regulate EGFR and apoptosis in glioblastoma. *Cancer Res.* 2014; 74:1541–1553. [PubMed: 24425048]
36. Heo MJ, Kim YM, Koo JH, Yang YM, An J, Lee SK, et al. microRNA-148a dysregulation discriminates poor prognosis of hepatocellular carcinoma in association with USP4 overexpression. *Oncotarget.* 2014; 5:2792–2806. [PubMed: 24798342]
37. Li L, Liu Y, Guo Y, Liu B, Zhao Y, Li P, et al. Regulatory MiR-148a-ACVR1/BMP circuit defines a cancer stem cell-like aggressive subtype of hepatocellular carcinoma. *Hepatology.* 2015; 61:574–584. [PubMed: 25271001]
38. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *Cell.* 2008; 132:344–362. [PubMed: 18267068]
39. Ammirante M, Kuraishy AI, Shalapour S, Strasner A, Ramirez-Sanchez C, Zhang W, et al. An IKKalpha-E2F1-BMI1 cascade activated by infiltrating B cells controls prostate regeneration and tumor recurrence. *Genes Dev.* 2013; 27:1435–1440. [PubMed: 23796898]
40. Jiang R, Xia Y, Li J, Deng L, Zhao L, Shi J, et al. High expression levels of IKKalpha and IKKbeta are necessary for the malignant properties of liver cancer. *Int J Cancer.* 2010; 126:1263–1274. [PubMed: 19728335]
41. Xiao Z, Jiang Q, Willette-Brown J, Xi S, Zhu F, Burkett S, et al. The pivotal role of IKKalpha in the development of spontaneous lung squamous cell carcinomas. *Cancer Cell.* 2013; 23:527–540. [PubMed: 23597566]
42. Zhang J, Jiao J, Cermelli S, Muir K, Jung KH, Zou R, et al. miR-21 inhibition reduces liver fibrosis and prevents tumor development by inducing apoptosis of CD24+ progenitor cells. *Cancer Res.* 2015; 75:1859–1867. [PubMed: 25769721]

43. Descargues P, Sil AK, Karin M. IKK α , a critical regulator of epidermal differentiation and a suppressor of skin cancer. *EMBO J.* 2008; 27:2639–2647. [PubMed: 18818691]
44. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science.* 1999; 284:770–776. [PubMed: 10221902]
45. Jeliaskova P, Jors S, Lee M, Zimmer-Strobl U, Ferrer J, Schmid RM, et al. Canonical Notch2 signaling determines biliary cell fates of embryonic hepatoblasts and adult hepatocytes independent of Hes1. *Hepatology.* 2013; 57:2469–2479. [PubMed: 23315998]
46. Geisler F, Strazzabosco M. Emerging roles of Notch signaling in liver disease. *Hepatology.* 2015; 61:382–392. [PubMed: 24930574]
47. Dill MT, Tornillo L, Fritzius T, Terracciano L, Semela D, Bettler B, et al. Constitutive Notch2 signaling induces hepatic tumors in mice. *Hepatology.* 2013; 57:1607–1619. [PubMed: 23175466]
48. Huntzicker EG, Hotzel K, Choy L, Che L, Ross J, Pau G, et al. Differential effects of targeting Notch receptors in a mouse model of liver cancer. *Hepatology.* 2015; 61:942–952. [PubMed: 25311838]
49. Villanueva A, Alsinet C, Yanger K, Hoshida Y, Zong Y, Toffanin S, et al. Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice. *Gastroenterology.* 2012; 143:1660–1669. e1667. [PubMed: 22974708]
50. Zender S, Nickeleit I, Wuestefeld T, Sorensen I, Dauch D, Bozko P, et al. A critical role for notch signaling in the formation of cholangiocellular carcinomas. *Cancer Cell.* 2013; 23:784–795. [PubMed: 23727022]

Fig. 1.

Identification of miRNAs associated with hepatocytic differentiation and HCC. (A) List of miRNAs that are modulated during hepatocytic differentiation of HepaRG liver progenitor cells and inversely modulated in Huh7 liver cancer cells. (B) Expression of identified miRNAs listed in (A) based on microarray analysis from primary HCCs (Tumor) and histologically noncancerous liver tissues (Non-Tumor) from 96 patients (accession number: GSE22058). Gene expression levels ($\log_2$ intensity) were assigned for these categories and represented as a scatter plot, and the median expression level of each group was indicated by horizontal line. The differential miRNA expression in HCCs was determined by the unpaired Student t-test (mean $\pm$ SEM, versus Non-Tumor).

Fig. 2. Overexpression of miR-148a accelerates hepatocytic differentiation of HepaRG cells. (A) Expression of selected miRNAs was determined by quantitative PCR in HepaRG-Empty and individual HepaRG-miRNA clones (miR-18a, miR-29c, miR-93, miR-101, miR-148a, miR-150 and miR-221). The graphs show fold-changes and SEM (unpaired Student t test, versus HepaRG-Empty). (B) Images of HepaRG-Empty and individual HepaRG-miRNA clones were taken at different time-points during the differentiation process: progenitor cells (day 2), intermediate (day 14) and hepatocyte-like cells (day 28). Scale Bars, 50 μ m. (C) Mean bile canaliculi densities assessed from three randomly selected fields in two independent experiments at day 14 and day 28 of hepatocytic differentiation in HepaRG-Empty and individual HepaRG-miRNA clones. Data are presented as the means $\pm$ SEM (unpaired Student t test, versus HepaRG-Empty). (D) Expression of HNF4A, ALDOB, ALB, CYP3A4 and miR-122 at day 14 of the differentiation process in HepaRG-miR-148a cell lines compared to HepaRG-Empty cell lines. The graphs show fold-changes and SEM in two independent experiments.

Fig. 3.

Effect of miR-148a treatment on tumor growth, tumor phenotype and liver fibrosis. (A) Liver of mice from placebo and miR-148a treated groups were imaged with MRI to evaluate the tumor growth during treatment. Representative tumors identified by MRI are indicated by yellow arrows. (B) Tumor growth expressed as fold change over 2 weeks of treatment, in placebo treated and miR-148a treated groups as determined by MRI. Data are presented as the means $\pm$ SEM (unpaired Student t test, versus placebo treated group). (C) Tumor size distribution in each treatment group. The tumors are separated into $\leq 50 \text{ mm}^3$ or $> 50 \text{ mm}^3$. Average percentages of tumors are represented on the Y-axis and tumor size categories on the X-axis. The graphs show as mean $\pm$ SEM (unpaired Student t test, versus placebo treated group). (D) Pie charts representing the overall tumor histology distribution within the two treatment groups. (E) Liver fibrosis was measured by Masson's Trichrome staining. Representative pictures of liver fibrosis are showed in the left panel. Scale Bars, $50 \mu\text{m}$. The fibrosis was quantified by determining the percentage of the positive staining area (blue staining) out of the whole liver tissue area (mean $\pm$ SEM, unpaired Student t test, versus placebo treated group).

Fig. 4. Overexpression of miR-148a promotes hepatocytic differentiation phenotype in vivo. (A, B and C) Expression of progenitor markers CD24 and OPN, biliary cell markers KRT19 and SOX9 and hepatocyte markers HNF4A and miR-122, was measured by quantitative PCR in tumors of placebo treated and miR148a treated mice. The results show as mean $\pm$ SEM (unpaired Student t test, versus placebo treated group). (D) Co-immunofluorescence staining for anti-EPCAM and anti-A6 antibodies on liver sections from placebo treated and miR-148a treated mice. Scale Bars, 100 μ m. The ratio of A6/EPCAM is shown (right panel). Data are represented as mean $\pm$ SEM (unpaired Student t test, versus placebo treated group).

Fig. 5.

MiR-148a treatment prevents tumor development and reduces liver fibrosis. (A) Effect of miR-148a treatment on tumor incidence. (B) Graph showing relative average tumor volume and burden in placebo treated and miR-148a treated mice. Tumor sizes were measured with calipers in three dimensions at necropsy. Tumor volumes were calculated using the formula: Tumor volume (mm³) = $L \times W^2 / 2$, where L is length and W is width. Graphs show mean $\pm$ SEM and data were analyzed by Student's t test (versus placebo treated group). (C) Pie charts showing the tumor histology distribution in placebo treated and miR-148a treated mice. (D) The graph shows the quantitative analysis of liver fibrosis in placebo treated and miR-148a treated group. The Fibrotic scoring was quantified by determining the percent of positive staining area (blue staining) out of the whole tissue area. The graph bars represent means $\pm$ SEM (unpaired Student t test, versus placebo treated group). (E) Serum aspartate aminotransferase (AST) activity was measured in the serum of placebo treated and miR-148a treated mice. Values represent mean $\pm$ SEM (unpaired Student t test, versus placebo treated group).

Fig. 6.

IKKα is a direct target of miR-148a and IKKα silencing induces hepatocytic differentiation of HepaRG cells while overexpression of NOTCH2 suppresses miR-148a-induced hepatocytic differentiation in HepaRG cells. (A, left panel) Predicted miR-148a binding sites within the human and mouse IKKα-3'UTR are shown in complementary pairing. (A, middle panel) IKKα mRNA expression upon hepatocytic differentiation of liver progenitor HepaRG cells measured by quantitative PCR and shown as fold-changes and SEM, at day 28 relative to day 2, in two independent experiments. (A, right panel) Cells were transfected with IKKα-3'UTR reporter constructs (200 ng) in the presence of negative control miRNA (NC; 100 nM) or miR-148a (50 nM or 100 nM). Effects of miR-148a on the reporter constructs were determined at 48 h after transfection. Relative luciferase activities were measured and calculated as the ratio of firefly/renilla activities in the cells, and normalized to those of the negative control miRNA. The results were presented as means $\pm$ SEM from three independent experiments ($p < 0.05$). (B, left panel) IKKα mRNA levels measured by qRT-PCR in HepaRG-empty and HepaRG- IKKα KD cell lines. (B, middle panel) Bile canaliculi densities in HepaRG-empty and HepaRG- IKKα KD cell lines upon hepatocytic differentiation, assessed from three randomly selected fields in two independent experiments. (B, right panel) Relative mRNA expression of HNF4A, ALDOB and miR-122 at day 14 of hepatocytic differentiation process in HepaRG- IKKα KD compared to HepaRG-empty cell lines. (C, left panel) NOTCH2 and miR-148a expression levels

determined by qRT-PCR in HepaRG-empty, HepaRG-miR-148a and HepaRG-miR-148a-NOTCH2 cell lines. (C, middle panel) Bile canaliculi densities of HepaRG-Empty, HepaRG-miR-148a and HepaRG-miR-148a-NOTCH2 cell lines upon hepatocytic differentiation, assessed from three randomly selected fields in two independent experiments. (C, right panel) Relative mRNA expression of HNF4A, ALDOB and miR-122 measured by qRT-PCR at day 14 of the differentiation process in HepaRG-Empty, HepaRG-miR-148a-NOTCH2 and HepaRG-miR-148a cell lines.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 7.

RO4929097 treatment inhibits tumor growth and reduces tumor malignancy and liver fibrosis. (A) mRNA expression of NOTCH target gene HEY1 was measured by quantitative PCR. (B) Tumor growth expressed as fold change over 14 days of treatment, in placebo treated and RO4929097 treated groups as determined by MRI. Data are presented as the means $\pm$ SEM (unpaired Student t test, versus placebo treated group). (C) Tumor size distribution per mouse in each group. (D) Pie charts representing the tumor histology distribution with placebo treated and RO4929097 treated mice. (E) Representative images of Masson's trichrome stains for fibrosis (blue staining) are showed in left panel and quantification data was calculated by the percentage of positive staining areas. Scale Bars, 50 μm .

Fig. 8.

RO4929097 treatment prevents tumor generation and decreased liver fibrosis. (A) Effect of RO4929097 treatment on tumor incidence. (B) Graph showing relative average tumor volume and burden in placebo treated and RO4929097 treated mice. Tumor sizes were measured with calipers in three dimensions at necropsy. Graphs show mean $\pm$ SEM and data were analyzed by Student's t test (versus placebo treated group). (C) Pie charts showing the overall tumor type distribution in placebo treated and RO4929097 treated mice by histopathological examination. (D) Measurement of liver fibrosis by Masson's trichrome staining in both treatment groups.