Published in final edited form as:

Cancer Res. 2015 May 1; 75(9): 1859–1867. doi:10.1158/0008-5472.CAN-14-1254.

MiR-21 Inhibition Reduces Liver Fibrosis and Prevents Tumor Development by Inducing Apoptosis of CD24+ Progenitor Cells

Jing Zhang¹, Jingjing Jiao¹, Silvia Cermelli², Kyle Muir², Kwang Hwa Jung¹, Ruhai Zou^{1,3}, Asif Rashid⁴, Mihai Gagea⁵, Sonya Zabludoff⁶, Raghu Kalluri⁷, and Laura Beretta^{1,2}

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

²Molecular Diagnostics Program, Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA.

³State Key Laboratory of Oncology in Southern China; Department of Ultrasound, Sun Yat-sen University Cancer Center, Guangzhou, P. R. China 510060

⁴Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA.

⁵Department of Veterinary Medicine & Surgery, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA.

⁶Regulus Therapeutics, Inc, San Diego, CA 92121, USA.

⁷Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA.

Abstract

MiR-21 is upregulated in hepatocellular carcinoma and intrahepatic cholangiocarcinoma where it is associated with poor prognosis. Here we offer preclinical evidence that miR-21 offers a therapeutic and chemopreventive target in these liver cancers. In mice with hepatic deletion of *Pten*, anti-miR-21 treatment reduced liver tumor growth and prevented tumor development. These effects were accompanied with a decrease in liver fibrosis and a concomitant reduction of CD24+ liver progenitor cells and S100A4+ cancer-associated stromal cells. Notch2 inhibition also occurred in tumors following anti-miR-21 treatment. We further showed that miR-21 is necessary for the survival of CD24+ progenitor cells, a cellular phenotype mediated by Notch2, osteopontin and integrin αv . Our results identify miR-21 as a key regulator of tumor-initiating cell survival, malignant development and growth in liver cancer, highlighting the role of CD24+ cells in expansion of S100A4+ cancer-associated stromal cells and associated liver fibrosis.

Corresponding author: Laura Beretta, 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.

Disclosures: S.Z. is a full-time employee of Regulus Therapeutics. Other authors have nothing to disclose.

Keywords

HCC prevention; miR-21; CD24+ progenitor cells; osteopontin

Introduction

MicroRNAs (miRNA) are small non-coding RNA molecules that affect mRNA stability and translation by targeting the 3'-untranslated region (3'-UTR) of various transcripts. Dysregulation of miRNAs affects a wide range of cellular processes including cell proliferation and differentiation. Interest in microRNA-21 (miR-21) has increased recently, especially in cancer and cardiovascular diseases (1). MiR-21 is up-regulated in almost all types of cancers, promotes cancer cell proliferation, migration, and survival and therefore was classified as an onco-miR (2, 3). High levels of miR-21 are often associated with aggressive forms of cancer and poor patient survival. MiR-21 is significantly up-regulated in hepatocellular carcinoma (HCC) and is associated with poor prognosis (4-10). MiR-21 was also reported to be overexpressed in intrahepatic cholangiocarcinoma and cholangiocarcinoma but no correlation with clinicopathological features was found (10, 11). A role of miR-21 in organ fibrosis has also been described (1). MiR-21 regulates epithelial-mesenchymal transition (EMT), a process in which fibroblasts derive from epithelial or endothelial cells resulting in organ fibrosis. A direct evidence for a role of miR-21 in organ fibrosis was obtained from studies in mouse models of cardiac and pulmonary fibrosis (12, 13). Up-regulation of miR-21 was found in human fibrotic livers resulting chronic hepatitis C virus (HCV) infection and intrahepatic miR-21 levels positively correlated with HCV viral load, fibrosis severity or serum liver transaminase levels (14). Cirrhosis, the result of end-stage fibrosis, is a common pre-neoplastic condition associated with hepatocarcinogenesis (15). It is therefore highly relevant to study the role of miR-21 in hepatocarcinogenesis. Deletion of the phosphoinositide 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN) in hepatocytes leads to steatohepatitis, fibrosis, hepatocyte dysplasia and HCC later in life, reproducing the steps of HCC development observed in human HCC (16, 17). It therefore represents an excellent model to evaluate the potential of targeting miR-21 for the treatment and chemoprevention of HCC.

Materials and Methods

Mice treatment

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⁺*. Only male mice were included in this study as female mice develop tumors at a much lower incidence rate. Two separate sets of treatment were performed. In the first set, 12 × 10.5 month-old male *Pten* null mice received anti-miR-21 (25 mg/kg) in phosphate-buffered saline (PBS) carriage medium or placebo by intraperitoneal injection. In the second set, 12 × 7.5 month-old male *Pten* null mice received anti-miR-21 (25 mg/kg) or placebo by intraperitoneal injection. The anti-miR-21 used was a high-affinity oligonucleotide complementary to the active site of miR-21 with a phosphorothioate backbone containing modifications (DNA, MOE, cET) (Regulus

Therapeutics). The PK of these chimeric phosphorothioate antisense oligonucleotides is independent of the sequence. The compounds PK and pharmacodynamics across species (mice, primates, human) together with their safety profiles and dose-dependent actions in liver, the major organ of deposition, have been described (18). All mice received eight injections over a period of six weeks, three injections in the first week of treatment and one injection per week in the following five weeks. C57BL/6 wild type (wt) and OPN knockout (OPN^{-/-}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). A total of 7 × 10 week-old male wt mice and 6 × 10 week-old male OPN^{-/-} mice were fed with either normal diet or a diet supplemented with 0.1% diethoxycarbonyl-1, 4-dihydro-collidin (DDC) (Sigma-Aldrich) for 4 weeks. All animal studies were carried out in strict accordance with institutional regulations and every effort was made to minimize the number of animals required for the study and to minimize the pain and discomfort experienced.

miR-21 *in situ* hybridization and immunofluorescence co-staining

Formalin-fixed paraffin embedded tissue sections were deparaffinized in xylene, and rehydrated using ethanol dilutions. For miR-21 *in situ* hybridization, tissue sections were digested with 5 µg/mL proteinase K for 5 minutes at room temperature, then loaded onto Ventana Discovery Ultra for *in situ* hybridization analysis. The tissue slides were incubated with double-DIG labeled mercury LNA microRNA probe (exiqon) for 2 hours at 55°C. The digoxigenins were then detected with a polyclonal anti-DIG antibody and Alkaline Phosphatase conjugated second antibody (Ventana) using NBT-BCIP as the substrate. Negative microRNA probe from Exiqon was used as negative control. Positive control was performed using microRNA U6. For co-staining, microRNA probe labeled slides were treated with 3% H₂O₂ to inactivate endogenous peroxidase and blocked with 5% bovine serum albumin in PBS (w/v). OPN (R&D) primary antibody was used followed by secondary antibody incubation in PBST and tyramine conjugated fluorochrome.

Apoptosis assays

In vitro cell apoptosis was tested using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) following transfection with 20nM oligonucleotide (hsa-miR-21 Anti-miR from Ambion Life Technologies) for 72 hrs using 10µL Lipofectamine RNAiMAX transfection reagent. For OPN rescue assays, 1µg/mL of recombinant OPN protein (R&D) was added to the culture medium following anti-miR-21 transfection. For ITGAV blocking experiments, 5µg/mL of ITGAV blocking antibody (Abcam) was added to the culture medium. Same amount of IgG was used as negative control.

Additional methods are provided in Supplementary Methods

Results

In mice with hepatocytic deletion of *Pten*, miR-21 expression is increased in liver tumors, correlates with fibrosis in adjacent liver and is enriched in progenitor cells

Hepatic deletion of *Pten* in male mice induced liver steatosis at around 3 month and steatosis severity increased with aging, remaining stable after 6 month (Supplementary Fig. S1A). Mild liver fibrosis was detected in 6-month-old *Pten* null mice and gradually increased in

severity with aging (Supplementary Fig. S1B). At 9-month-old, around 80% of the *Pten* null mice had developed tumors and all 12-month-old mice presented with tumors (Supplementary Fig. S1C). We measured the expression of miR-21 in the liver and tumors of these mice. MiR-21 levels were not statistically different in control healthy liver and in steatotic liver from 6 month-old *Pten* null mice (median of 3.7×10^9 copies and 2.9×10^9 copies, respectively). MiR-21 expression significantly increased in liver of 9- and 12-month-old *Pten* null mice (median = 7.99×10^9 copies, $p < 0.001$) and further increased in tumors (median = 13.0×10^9 copies, $p = 0.02$) (Fig. 1A). Fibrosis and lipid deposition were measured by histology in all mice and the size of the tumors was recorded. While levels of miR-21 didn't correlate with tumor size or steatosis levels, miR-21 expression strongly correlated with fibrosis severity ($R = 0.71$) (Fig. 1B).

By *in situ* hybridization of miR-21 in liver and tumors of *Pten* null mice, we didn't detect any miR-21 expression in hepatocytes nor HCC cells. Instead, a strong positive miR-21 signal was detected in ductular reaction areas in the liver, in areas surrounding the tumors and in neoplastic biliary cells (Fig. 1C). More specifically, miR-21 was enriched in cells expressing osteopontin (OPN, *Spp-1*), a marker of hepatic progenitor cells and biliary cells (Fig. 1D). To further validate the relevance of this expression pattern in human disease, *in situ* hybridization of miR-21 was performed on six human resected HCCs. As observed in the *Pten* null mouse model, miR-21 was mainly expressed in areas surrounding the tumors and in non-neoplastic bile ducts. The tumor areas were negative in five HCCs and partially positive in the sixth one (Fig. 1E).

Anti-miR-21 treatment inhibits tumor growth, modifies the tumor differentiation phenotype and reduces liver fibrosis

To evaluate the therapeutic potential of targeting miR-21, we treated 10.5-month-old *Pten* null mice for 6 weeks, with chemically modified antisense oligonucleotides specific for miR-21. At necropsy, tumors and adjacent tissues were collected and processed for gene expression and histology analyses. We first validated the anti-miR-21 treatment efficiency by measuring miR-21 target genes *Sprouty-1* and *Sprouty-2* (*Spry1* and *Spry2*). Upon anti-miR-21 treatment, expression of *Spry1* and *Spry2* significantly increased (2.1-fold; $p = 0.032$ and 2.0-fold; $p = 0.043$, respectively), confirming that the anti-miR-21 treatment was effective in reducing miR-21 activity (Fig. 2A). We next evaluated the effect of anti-miR-21 on tumor growth (Fig. 2B-D). Anti-miR-21 treatment decreased the ratio of liver/body weight from 23.4% to 20.1% ($p = 0.028$) (Fig. 2B). While anti-miR-21 treatment didn't affect the average number of tumors per mouse (2.7 and 2.8 for placebo and anti-miR-21 groups, respectively) (Fig. 2B), the average tumor burden in anti-miR-21 treated mice was significantly smaller than in placebo treated mice (891 mm^3 compared to 2308 mm^3 , $p = 0.05$) (Fig. 2C). The average tumor size in anti-miR-21 treated mice was also significantly smaller than in placebo treated mice (342 mm^3 compared to 865 mm^3 , $p = 0.05$) (Fig. 2C). The reduction in tumor size following anti-miR-21 treatment was further demonstrated by the tumor size distribution per mouse in both groups. While half of the tumors in placebo-treated mice were $>500 \text{ mm}^3$, only 21.7% of the tumors in anti-miR-21 treated mice were $>500 \text{ mm}^3$ (Fig. 2D).

Blinded evaluation by a pathologist of 14 tumors from the placebo group and 11 tumors from the anti-miR-21-treated group identified the placebo tumors as displaying multiple morphologic characteristics including hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA) and hepatocholangiocellular carcinoma. In contrast, anti-miR-21 treated tumors showed a significant reduction in the incidence of cholangiolar tumors with incidence of CCA and hepatocholangiocellular carcinoma decreasing from 29% and 71%, respectively in the placebo treated group to 9% and 27%, respectively in the anti-miR-21 treated group. In addition, anti-miR-21 treatment resulted in a decrease in the grade of tumor malignancy from predominantly pleomorphic and heterogeneous HCC to well-differentiated HCC. The incidence of pleomorphic HCC decreased from 42% in the placebo treated group to 27% in the anti-miR-21 treated group. The majority of tumors in anti-miR-21 treated group were well differentiated HCCs, suggesting that inhibition of miR-21 resulted not only in reduced tumor growth but also in histologically less malignant liver tumors. Representative images of hepatocholangiocellular carcinoma and pleomorphic HCC in the placebo treated group and of well-differentiated HCC in the anti-miR-21 treated groups are shown in Supplementary Fig. S2.

Finally, because of the strong correlation we observed between liver fibrosis and miR-21 expression, we also evaluated the effects of anti-miR-21 treatment on fibrosis in adjacent liver tissue. Masson's trichrome staining showed a significant reduction of fibrosis from 19.0% to 12.0% ($p=0.002$) upon anti-miR-21 treatment (Fig. 2E).

Anti-miR-21 treatment results in a significant reduction of progenitor cells and S100A4+ cancer-associated stromal cells

To determine the mechanisms by which anti-miR-21 inhibits tumor growth and liver fibrosis, we first evaluated the effects of anti-miR21 treatment on OPN expressing progenitor cells. A strong reduction in OPN⁺ cell population was observed following anti-miR-21 treatment. EPCAM, another marker of hepatic progenitor cells and biliary cells showed similar reduction. In addition, S100A4, a marker of cancer-associated stromal cells was also tested. Anti-miR-21 treatment resulted in a dramatic decrease of S100A4+ cells (Fig. 3A). These results were further validated by real-time PCR confirming a significant decrease in *Opn* and *Epcam* mRNA expression (−2.7-fold; $p=0.004$ and −2.9-fold; $p=0.012$ respectively). The expression of other hepatic progenitor markers *Krt7*, *Krt19* and of the stem cell marker *Prom1* was also significantly reduced upon anti-miR-21 treatment (−2.7-fold; $p=0.019$; −2.1-fold; $p=0.027$ and −2.3-fold; $p=0.028$, respectively). Finally, anti-miR-21 treatment resulted in a significant reduction of *S100A4* mRNA expression (−2.1-fold; $p=0.034$) (Fig. 3B). Together, these data showed that anti-miR-21 treatment results in a decrease in progenitor cell population, an effect accompanied with a decrease in S100A4+ cancer associated stromal cells.

MiR-21 is required for the survival of CD24+ cells: a mechanism mediated by osteopontin and integrin αv

To determine the mechanisms by which anti-miR-21 results in a decrease in progenitor cell population, we treated the human HepaRG liver progenitor cells with 20nM anti-miR-21 oligonucleotide. MiR-21 level was 3.5-fold higher in HepaRG cells compared to healthy

liver and hepatoma cells lines Huh7 and PLC/PRF5 (Fig. 4A) and anti-miR-21 treatment resulted in increased expression of miR-21 targets, *Spry1* and *Spry2* (2.2-fold; $p=0.015$ and 1.9-fold; $p=0.039$, respectively), validating the efficiency of the anti-miR-21 treatment in HepaRG cells (Fig. 4B). After 72hrs of anti-miR-21 treatment, an average of 38% of the HepaRG cells underwent apoptosis as shown by Annexin V/PI staining (Fig. 4C, upper panels). The same treatment in the hepatoma Huh7 cells didn't induce any apoptosis (Fig. 4C, lower panels). To further identify the liver progenitor cell subpopulation undergoing apoptosis upon miR-21 inhibition, we stained HepaRG cells with antibodies directed against two commonly used liver stem cell surface markers CD44 and CD24. Approximately 54% of HepaRG cells expressed CD24 while 99% of HepaRG cells expressed CD44 (Fig. 4D). Upon miR-21 inhibition, *CD24* mRNA expression in HepaRG cells decreased (-2.13 fold; $p<0.001$) while *CD44* mRNA expression slightly increased, suggesting a specific effect of miR-21 on CD24+ cells (Fig. 4E). Co-staining of CD24 and Annexin V further showed that the large majority (75%) of apoptotic cells resulting from miR-21 inhibition were CD24+ cells (Fig. 4F). The specific effect of miR-21 inhibition on the CD24+ subpopulation of liver progenitor cells was further confirmed *in vivo*, by immunostaining showing a significant reduction of CD24+ cells in *Pten* null liver treated with anti-miR-21 from 12.9% to 4.1% ($p=0.013$) (Fig. 4G). In conclusion, miR-21 is required for the survival of CD24+ liver stem cells. To further evaluate whether CD24 is indeed a stem-like marker, we separated HepaRG cells into CD24+ and CD24- populations by fluorescence activated cell sorting and subjected them to sphere formation assays. CD24+ cells formed significantly larger spheres and at a significantly higher frequency as compared to CD24- cells (Fig. 4H).

We wished to identify the mediators of anti-miR-21 induced apoptosis of CD24+ cells. We failed to identify candidate targets of miR-21 with apoptotic functions and preferential effects on CD24+ cells. We therefore investigated the possible involvement of external signaling effects. We measured the expression of integrin αv (ITGAV), a receptor for OPN. Over 90% of HepaRG cells expressed ITGAV (Fig. 5A). To evaluate whether ITGAV signaling is required for CD24+ cells survival, HepaRG cells were treated with 5 μ g/mL ITGAV neutralizing antibody for 72 hrs. This treatment resulted in the apoptosis of 34.5% HepaRG cells. Among those apoptotic cells, 77% were CD24+ cells (Fig. 5B). Real-time PCR also showed that ITGAV neutralizing antibody treatment caused a strong (-3.7-fold; $p<0.001$) decrease in CD24 mRNA expression (Fig. 5C). We then added 1 μ g/mL of recombinant OPN protein to the cell culture medium following anti-miR-21 transfection. Addition of OPN partially blocked the anti-miR-21 induced apoptosis of HepaRG cells, decreasing apoptosis from 29.87% to 20.25% ($p=0.046$) (Fig. 5D). Together, these results suggest that OPN is required for the survival of CD24+ cells, an effect mediated by ITGAV. To further validate the role of OPN on CD24+ cells survival, OPN knockout (OPN^{-/-}) mice were challenged with 4-weeks DDC diet, a classic liver injury model which strongly induces liver progenitor cell proliferation. DDC diet dramatically enhanced CD24 mRNA expression (39.7-fold) in wild type (wt) mice. In OPN^{-/-} mice, DDC-induced CD24 expression was significantly reduced ($p=0.021$) (Fig. 5E).

Inhibition of *Notch2* in *Pten* null tumors upon anti-miR-21 treatment

To further identify the upstream events leading to OPN inhibition by anti-miR-21, we investigated whether *Notch* expression and activity were increased in *Pten* null liver and tumors and whether anti-miR-21 treatment affected *Notch* expression and activity. The *Notch* family members have been associated with biliary differentiation of hepatoblast, liver carcinogenesis and liver fibrosis. In addition, Opn expression is directly regulated by the transcriptional factor Runx2, a Notch target. *Runx2* was significantly increased in *Pten* null tumors compared to adjacent liver (1.5-fold, $p=0.011$) and anti-miR-21 treatment resulted in a -1.5 -fold reduction of *Runx2* mRNA in tumors ($p=0.009$). Similarly, the expression of another Notch target, *Hes1* was increased in *Pten* null tumor compared to adjacent liver (1.7-fold; $p=0.019$) and strongly reduced upon anti-miR-21 treatment (-3.3 -fold; $p<0.001$) (Fig. 6A). These results are indicative of increased *Notch* activity in *Pten* null tumors that can be inhibited by anti-miR-21 treatment. We then measured the expression of *Notch 1*, *2*, *3* and *4*. While the expression of *Notch1* was not increased in tumors compared to adjacent liver, the expression of *Notch2*, *Notch3* and *Notch4* was significantly increased in tumors (2.2-fold; $p=0.007$; 6.0-fold; $p=0.005$; 1.5-fold; $p=0.001$ respectively). Anti-miR-21 significantly reduced *Notch1* and *Notch3* expression in the liver ($p=0.021$ and $p=0.013$, respectively) but had no effect on their expression in tumors. In contrast, anti-miR-21 treatment resulted in a reduction of *Notch2* in tumors (-1.8 fold, $p=0.042$). No effect of anti-miR-21 treatment on *Notch4* was observed (Fig. 6B). Because anti-miR-21 targets hepatic progenitor cells, we evaluated whether *Notch2* was preferentially expressed in these cells. In HepaRG cells, *NOTCH2* is the major *NOTCH* gene, followed by *NOTCH1*. *NOTCH3* and *NOTCH4* mRNAs are expressed at very low levels. Anti-miR-21 treatment of HepaRG cells resulted in a concomitant decrease in *NOTCH2* and *OPN* expression (-1.6 -fold; $p=0.015$ and -2.8 -fold; $p=0.006$, respectively) (Fig. 6C). We further showed that *NOTCH2* downregulation by shRNA in HepaRG cells resulted in *RUNX2* and *OPN* downregulation (Fig. 6D). Together, these results showed that *Notch2* is enriched in progenitor cells and mediates the down regulation of *OPN* by anti-miR-21 treatment.

Anti-miR-21 treatment prevents tumor development

Because of the effects of miR-21 inhibition on CD24+ cell survival, OPN levels, expansion of S100A4+ stromal cells and *Notch* activity, we investigated whether targeting miR-21 could prevent tumor development *in vivo* in *Pten* null mice. We treated 7.5-month-old *Pten* null mice with anti-miR-21 for 6 weeks. Ultrasound didn't detect any tumor in these mice at the start of treatment. At the end of treatment, the incidence of histologically confirmed tumors was 67% in the placebo treated group and 33% in the anti-miR-21 treated group (Fig. 7A). The average tumor burden in anti-miR-21-treated mice was also significantly smaller than in mice from the placebo treated group (23 mm^3 compared to 107 mm^3 , $p=0.039$) (Fig. 7B). While the average volume of the largest tumor in mice from the placebo group was 92 mm^3 , the average volume of the largest tumor in mice from the anti-miR-21 group was only 18 mm^3 ($p=0.05$) (Fig. 7C). Overall, only one tumor in the anti-miR-21 treated group was over 20 mm^3 . Ultrasound analysis also showed that anti-miR-21 treatment significantly reduced the tumor growth rate from 2.24-fold to 1.25-fold over 14 days

($p=0.029$) (Fig. 7D). As observed in the first set of *Pten* null treated mice, fibrosis was significantly reduced from 14.3% to 7.2% ($p=0.014$) upon anti-miR-21 treatment (Fig. 7E).

Discussion

MiR-21 has been identified as an onco-miR associated with many types of cancers including hepatocellular carcinoma and intrahepatic cholangiocarcinoma (10, 19). MiR-21 promotes cancer cell proliferation and invasion, and prevents apoptosis through the regulation of its target genes (8, 20, 21). The study presented here is the first evaluation of miR-21 as a therapeutic and preventive target in a genetically engineered mouse model of cancer. We found miR-21 expression in liver to be enriched in progenitor cells. Furthermore, we showed that a subpopulation of these progenitor cells, CD24+ cells, is dependent on miR-21 for their survival, an effect mediated by OPN-ITGAV signaling, and that anti-miR-21 treatment reduced CD24+ progenitor cell population *in vivo* by inhibiting RUNX2-mediated transcriptional regulation of OPN expression. CD24+ cells have been identified as liver tumor initiating cells and have been associated with higher risk of tumor recurrence (22). CD24 is an important molecule for stem cell self-renewal and tumor initiation ability through regulation of STAT3 phosphorylation and NANOG expression (22, 23). STAT3 can promote miR-21 expression by direct binding to the promoter region of miR-21 (24-27). It is therefore likely that the high expression of miR-21 in CD24+ cells we observed is mediated by STAT3.

The reduction in tumor incidence and growth following anti-miR-21 treatment *in vivo* may be the direct consequence of the depletion of CD24+ cell population. MiR-21 may mediate the early events in liver tumor initiation by maintaining the survival of CD24+ liver tumor initiating cells. This reduction in stem cell population may also explain the reduced malignancy of the tumors and concomitant increased differentiated phenotype following anti-miR-21 treatment. The increased differentiated phenotype following anti-miR-21 treatment may also result from the inhibition in *Notch* activity we observed. *Notch* signaling regulates biliary differentiation of hepatoblast in the developing and adult liver (28), drives cholangiocarcinoma development through transdifferentiation of hepatocytes (28-30) and contributes to liver carcinogenesis (31-33). *Notch* signaling has also been reported to modulate hepatic fibrosis (34). We found that *Notch2* is the main *Notch* family member expressed in *Pten* null liver tumors. This preferential expression of *Notch2* may be due to the predominant expression of *Notch2* in CD24+ progenitor stem cells. While anti-miR-21 treatment decreased *Notch1* expression in adjacent liver, *Notch2* expression decreased in tumors following anti-miR-21 treatment. This observation suggests that the different members of *Notch* have different cell distribution and non-redundant functions in liver.

The reduction in tumor incidence and growth following anti-miR-21 treatment *in vivo* may also be the direct consequence of a reduction in fibrosis and changes in the stroma largely associated with depletion of S100A4+ and of CD24+ cells, and reduction in OPN levels. A role for miR-21 in renal and cardiac fibrosis has been previously reported (1, 12, 35). Although miR-21 has been reported increased upon profibrogenic stimulation in liver (36), this is the first report demonstrating a direct role of miR-21 in liver fibrosis with a strong positive correlation between miR-21 levels and fibrosis severity in the *Pten* null liver and a

strong anti-fibrotic effect of anti-miR-21 treatment. In this model, we didn't observe any correlation between α -SMA levels, a stellate cell marker and the effect of anti-miR-21 on fibrosis. Instead the expression of S100A4 decreased following anti-miR-21 treatment. This decrease was associated with a strong reduction of osteopontin, an extracellular matrix protein known to be involved in liver fibrosis and early stages of liver tumor development (37, 38). Crosstalk between CD24⁺ cells and S100A4⁺ cells may contribute to the extension of fibrosis in the liver and anti-miR-21 treatment may reduce liver fibrosis by blocking this crosstalk.

In summary, this study provides *in vivo* evidence of a role for miR-21 in maintaining the survival of CD24⁺ progenitor cells and of a crosstalk between progenitor cells and cancer-associated stromal cells. It also suggests that anti-miR-21 may be effective at targeting tumor initiating cells as well as the tumor microenvironment, and therefore shows great promise for clinical studies of liver cancer prevention and treatment. The results of the study are summarized in a model diagram (Fig. S3). Further study is warranted to determine whether anti-miR-21 treatment can be effective as a companion therapeutic agent to drugs killing the tumor cells or as a therapeutic target for the prevention of liver tumor in patients at risk and the prevention of recurrence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Eric Marcusson and Deidre MacKenna from Regulus Therapeutics for expert assistance with targeting miR-21 *in vivo*.

Financial Support: This research is supported in part by the MD Anderson Cancer Center Support Grant CA016672, through a Multidisciplinary Research Program Award.

References

1. Kumarswamy R, Volkmann I, Thum T. Regulation and function of miRNA-21 in health and disease. *RNA Biol.* 2011; 8:706–13. [PubMed: 21712654]
2. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer.* 2006; 6:857–66. [PubMed: 17060945]
3. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. *Nat Rev Drug Discov.* 2010; 9:775–89. [PubMed: 20885409]
4. Jiang J, Gusev Y, Aderca I, Mettler TA, Nagorney DM, Brackett DJ, et al. Association of MicroRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. *Clin Cancer Res.* 2008; 14:419–27. [PubMed: 18223217]
5. Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology.* 2008; 47:1955–63. [PubMed: 18433021]
6. Burchard J, Zhang C, Liu AM, Poon RT, Lee NP, Wong KF, et al. microRNA-122 as a regulator of mitochondrial metabolic gene network in hepatocellular carcinoma. *Mol Syst Biol.* 2010; 6:402. [PubMed: 20739924]
7. Sato F, Tsuchiya S, Meltzer SJ, Shimizu K. MicroRNAs and epigenetics. *Febs J.* 2011; 278:1598–609. [PubMed: 21395977]

8. Gramantieri L, Fornari F, Callegari E, Sabbioni S, Lanza G, Croce CM, et al. MicroRNA involvement in hepatocellular carcinoma. *J Cell Mol Med*. 2008; 12:2189–204. [PubMed: 19120703]
9. Mizuguchi Y, Mishima T, Yokomuro S, Arima Y, Kawahigashi Y, Shigehara K, et al. Sequencing and bioinformatics-based analyses of the microRNA transcriptome in hepatitis B-related hepatocellular carcinoma. *PloS one*. 2011; 6:e15304. [PubMed: 21283620]
10. Karakatsanis A, Papaconstantinou I, Gazouli M, Lyberopoulou A, Polymeneas G, Voros D. Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. *Mol Carcinog*. 2013; 52:297–303. [PubMed: 22213236]
11. Selaru FM, Olaru AV, Kan T, David S, Cheng Y, Mori Y, et al. MicroRNA-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3. *Hepatology*. 2009; 49:1595–601. [PubMed: 19296468]
12. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature*. 2008; 456:980–4. [PubMed: 19043405]
13. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, et al. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *J Exp Med*. 2010; 207:1589–97. [PubMed: 20643828]
14. Marquez RT, Bandyopadhyay S, Wendlandt EB, Keck K, Hoffer BA, Icardi MS, et al. Correlation between microRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection in humans. *Lab Invest*. 2010; 90:1727–36. [PubMed: 20625373]
15. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology*. 2007; 132:2557–76. [PubMed: 17570226]
16. 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; 113:1774–83. [PubMed: 15199412]
17. 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; 101:2082–7. [PubMed: 14769918]
18. Yu RZ, Lemonidis KM, Graham MJ, Matson JE, Crooke RM, Tribble DL, et al. Cross-species comparison of in vivo PK/PD relationships for second-generation antisense oligonucleotides targeting apolipoprotein B-100. *Biochem Pharmacol*. 2009; 77:910–9. [PubMed: 19056355]
19. Xu J, Wu C, Che X, Wang L, Yu D, Zhang T, et al. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *Mol Carcinog*. 2011; 50:136–42. [PubMed: 21229610]
20. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology*. 2007; 133:647–58. [PubMed: 17681183]
21. Zhou L, Yang ZX, Song WJ, Li QJ, Yang F, Wang DS, et al. MicroRNA-21 regulates the migration and invasion of a stem-like population in hepatocellular carcinoma. *Int J Oncol*. 2013; 43:661–9. [PubMed: 23708209]
22. Lee TK, Castilho A, Cheung VC, Tang KH, Ma S, Ng IO. CD24(+) liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation. *Cell Stem Cell*. 2011; 9:50–63. [PubMed: 21726833]
23. Liu AY, Cai Y, Mao Y, Lin Y, Zheng H, Wu T, et al. Twist2 promotes self-renewal of liver cancer stem-like cells by regulating CD24. *Carcinogenesis*. 2013
24. Bourguignon LY, Earle C, Wong G, Spevak CC, Krueger K. Stem cell marker (Nanog) and Stat-3 signaling promote MicroRNA-21 expression and chemoresistance in hyaluronan/CD44-activated head and neck squamous cell carcinoma cells. *Oncogene*. 2012; 31:149–60. [PubMed: 21685938]
25. Kohanbash G, Okada H. MicroRNAs and STAT interplay. *Semin Cancer Biol*. 2012; 22:70–5. [PubMed: 22210182]

26. Loffler D, Brocke-Heidrich K, Pfeifer G, Stocsits C, Hackermuller J, Kretschmar AK, et al. Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood*. 2007; 110:1330–3. [PubMed: 17496199]
27. Yang CH, Yue J, Fan M, Pfeffer LM. IFN induces miR-21 through a signal transducer and activator of transcription 3-dependent pathway as a suppressive negative feedback on IFN-induced apoptosis. *Cancer Res*. 2010; 70:8108–16. [PubMed: 20813833]
28. 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; 57:2469–79. [PubMed: 23315998]
29. Sekiya S, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. *J Clin Invest*. 2012; 122:3914–8. [PubMed: 23023701]
30. Fan B, Malato Y, Calvisi DF, Naqvi S, Razumilava N, Ribback S, et al. Cholangiocarcinomas can originate from hepatocytes in mice. *J Clin Invest*. 2012; 122:2911–5. [PubMed: 22797301]
31. Dill MT, Tornillo L, Fritzius T, Terracciano L, Semela D, Bettler B, et al. Constitutive Notch2 signaling induces hepatic tumors in mice. *Hepatology*. 2013; 57:1607–19. [PubMed: 23175466]
32. Razumilava N, Gores GJ. Notch-driven carcinogenesis: The merging of hepatocellular cancer and cholangiocarcinoma into a common molecular liver cancer subtype. *J Hepatol*. 2013; 58:1244–5. [PubMed: 23352938]
33. 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; 143:1660–9. e7. [PubMed: 22974708]
34. Chen Y ZS, Qi D, Zheng S, Guo J, Zhang S, Weng Z. Inhibition of Notch signaling by a γ -secretase inhibitor attenuates hepatic fibrosis in rats. *PLoS One*. 2013; 7:11.
35. He Y, Huang C, Li J. miR-21 is a Critical Therapeutic Target for Renal Fibrosis. *Cell Biochem Biophys*. 2013
36. Noetel A, Kwiecinski M, Elfimova N, Huang J, Odenthal M. microRNA are Central Players in Anti- and Profibrotic Gene Regulation during Liver Fibrosis. *Front Physiol*. 2012; 3:49. [PubMed: 22457651]
37. Nagoshi S. Osteopontin: Versatile modulator of liver diseases. *Hepatol Res*. 2014; 44:22–30. [PubMed: 23701387]
38. Shang S, Plymoth A, Ge S, Feng Z, Rosen HR, Sangrajrang S, et al. Identification of osteopontin as a novel marker for early hepatocellular carcinoma. *Hepatology*. 2012; 55:483–90. [PubMed: 21953299]

Figure 1. miR-21 expression and cellular distribution in *Pten* null mice

A, Expression of miR-21 in liver from the *Pten* null mice expressed as copies per μg of total RNA. B, Correlation between miR-21 expression and liver fibrosis calculated using Pearson correlation analysis. C, miR-21 *in situ* hybridization: the dark-blue staining shows miR-21 positive cells present in areas of ductular reaction within the adjacent liver (left panel), surrounding the tumors (middle panel), and within cholangiolar tumors (right panel), magnification 100x. D, Immunofluorescence costaining of miR-21 and OPN in areas of ductular reaction within the adjacent liver (left panel), in areas surrounding the tumors (middle panel) and within cholangiolar hyperplasia (right panel), scale bar=100 μm . E, H&E staining and miR-21 *in situ* hybridization of a representative human HCC showing miR-21 staining in areas surrounding the tumor and lack of staining in the tumor.

Figure 2. Effect of anti-miR-21 treatment on tumor growth, tumor phenotype and liver fibrosis
 A, Hepatic expression of miR-21 target genes, *Spry1* and *Spry2*, in placebo and anti-miR-21 treated mice (n=6 mice per group) measured by qRT-PCR and shown as fold changes to the expression average in the placebo group. B, Average liver/body weight ratio and number of tumors per mouse detected at necropsy. C, Average tumor burden and tumor size per treatment group. D, Tumor size distribution per mouse in each group. E, Liver fibrosis measured by Masson's Trichrome staining. Representative staining pictures (40X magnification) are showed together with quantification corresponding to the percentage of positive staining areas in each mouse.

Figure 3. Reduction in progenitor and S100A4+ cell populations upon anti-miR-21 treatment
 A, Representative immunofluorescence tissue staining pictures (magnification 100X) for OPN, EPCAM and S100A4 in tumors of placebo and anti-miR-21 treated mice. B, mRNA expression of *OPN*, *Epcam*, *Krt7*, *Krt19*, *Prom1* and *S100A4* measured by qRT-PCR in tumors of both treatment groups.

Figure 4. Apoptosis of CD24+ cells following anti-miR-21 treatment

A, MiR-21 levels in healthy liver, hepatoma cell lines Huh7 and PLC/PRF5 and liver progenitor HepaRG cells. B, Expression of miR-21 target genes, *SPRY1* and *SPRY2* in HepaRG upon anti-miR-21 transfection. C, Progenitor HepaRG cells and Huh7 hepatoma cells were transfected with 20nM anti-miR-21 oligonucleotide for 72hrs and co-stained with Annexin V and PI. Cell apoptosis was analyzed by flow cytometry. The right panel shows the quantification data from three independent experiments. D, HepaRG cells were stained with CD24 and CD44 antibodies and CD24+ and CD44+ cell populations were characterized by flow cytometry. The right panel shows the quantification data from three independent experiments. E, Changes in *CD24* and *CD44* mRNA expression in HepaRG cells following anti-miR-21 transfection. F, HepaRG cells were transfected with anti-miR-21, co-stained with CD24 Antibody and Annexin V and then analyzed by flow cytometry. The right panel shows the quantification data from three independent experiments. G, Reduction in CD24+ progenitor cell population upon anti-miR-21 treatment of *Pten* null mice shown with representative immunofluorescence tissue staining pictures for CD24 (magnification 100X) and with quantification data calculated using the percentage of positive staining areas in liver from both treatment groups. H, Quantification of spheres formed by CD24+ and CD24- HepaRG cells.

Figure 5. Modulation of CD24+ cells survival by OPN and ITGAV

A, HepaRG cells were costained with CD24 and ITGAV antibodies and the positive populations were characterized by flow cytometry. B, Apoptosis of CD24+ cell population upon treatment with neutralizing ITGAV antibody. The right panel shows the quantification data from three independent experiments, 23 including % of apoptotic cells induced by ITGAV neutralizing antibody treatment and the distribution of these apoptotic cells between CD24- and CD24+ population. C, CD24 mRNA expression upon ITGAV neutralizing antibody treatment measured by qRT-PCR. D, HepaRG cells were treated with or without recombinant OPN protein (1 μ g/mL) following anti-miR-21 transfection. Cell apoptosis was analyzed at 72hrs by co-stained with Annexin V and PI. The figure shows the quantification data from three independent experiments. E, CD24 mRNA expression in liver from wt and OPN^{-/-} mice treated for 4 weeks with DDC.

Figure 6. Down-regulation of Notch upon anti-miR-21 treatment *in vivo* and *in vitro*
A-B, *Runx2*, *Hes1*, *Notch1*, *Notch2*, *Notch3* and *Notch4* mRNA expression measured by qRT-PCR in *Pten* null tumors and adjacent liver. C, Regulation of the *NOTCH* family members and *OPN* expression by anti-miR-21 treatment in HepaRG cells. D, *RUNX2* and *OPN* expression in HepaRG Notch2 stable knocking down cell lines measured by qRT-PCR.

Figure 7. Effect of anti-miR-21 treatment on liver fibrosis and tumor development

Effects of anti-miR-21 treatment on tumor incidence (A), average tumor burden per mouse (B), and largest tumor volume per mouse (C). D, Tumor growth expressed as fold change over 2 weeks of treatment, in placebo and anti-miR-21 treatment groups as determined by ultrasound. E, liver fibrosis measured by Masson's Trichrome staining.