Title

MicroRNA-21 and Dicer are dispensable for hepatic stellate cell activation and the development of liver fibrosis

Journal

Hepatology, 67(6)

ISSN

0270-9139

Authors

Caviglia, Jorge Matias
Yan, Jun
Jang, Myoung-Kuk
et al.

Publication Date

2018-06-01

DOI

10.1002/hep.29627

Peer reviewed

HHS Public Access

Author manuscript

Hepatology. Author manuscript; available in PMC 2019 June 01.

Published in final edited form as:

Hepatology. 2018 June ; 67(6): 2414–2429. doi:10.1002/hep.29627.

MicroRNA-21 and Dicer are Dispensable for Hepatic Stellate Cell Activation and the Development of Liver Fibrosis

Jorge Matias Caviglia[1], **Jun Yan**[1,2], **Myoung-Kuk Jang**[1,3], **Geum-Youn Gwak**[1,4], **Silvia Affo**[1], **Lexing Yu**[1], **Peter Olinga**[5], **Richard A. Friedman**[6], **Xin Chen**[7], and **Robert F. Schwabe**[1]

[1]Department of Medicine, Columbia University, New York, NY 10032, USA [2]Department of Pathology, Tianjin First Center Hospital, Tianjin, TJ 300192, China [3]Department of Gastroenterology and Hepatology, Internal Medicine, Kangdong Sacred Heart Hospital of Hallym University Medical Center, Seoul, 05355, South Korea [4]Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, 06351, Korea [5]Division of Pharmaceutical Technology and Biopharmacy, Department of Pharmacy, University of Groningen, 9700 AB Groningen, The Netherlands [6]Biomedical Informatics Shared Resource, Herbert Irving Comprehensive Cancer Center and Department of Biomedical Informatics, Columbia University, New York, NY 10032, USA [7]Department of Bioengineering and Therapeutic Sciences and Liver Center, University of California, San Francisco, CA, USA

Abstract

Fibrosis and cancer represent two major complications of chronic liver disease. MicroRNAs have been implicated in the development of fibrosis and cancer, thus constituting potential therapeutic targets. Here, we investigated the role of miR-21, a microRNA that has been implicated in the development of fibrosis in multiple organs and also been suggested to act as “oncomir”. Accordingly, miR-21 was the microRNA that showed the strongest upregulation in activated hepatic stellate cells (HSC) in multiple models of fibrogenesis, with an 8- to 24-fold induction compared to quiescent HSC. However, miR-21 antisense inhibition did not suppress the activation of murine or human HSC in culture or in liver slices. Moreover, antisense inhibition or genetic deletion of miR-21 in two independently generated knockout mice did not alter HSC activation or liver fibrosis in models of toxic and biliary liver injury. Despite a strong upregulation of miR-21 in injury-associated hepatocellular carcinoma and in cholangiocarcinoma, miR-21 deletion or antisense inhibition did not reduce the development of liver tumors. As inhibition of the most upregulated microRNA did not affect HSC activation, liver fibrosis and fibrosis-associated liver cancer, we additionally tested the role of microRNAs in HSC by HSC-specific Dicer deletion. Although Dicer deletion decreased microRNA expression in HSC and altered the expression of select genes, it only exerted negligible effects on HSC activation and liver fibrosis. In conclusion, genetic and pharmacologic manipulation of miR-21 does not inhibit the development of liver fibrosis and liver cancer. Moreover, suppression of microRNA synthesis does not significantly affect HSC phenotype and activation.

Correspondence to: Robert F. Schwabe, 1130 St. Nicholas Ave, ICRC, Room 926, New York, NY 10032; phone: 212-851-5462; rfs2102@cumc.columbia.edu; or to Jorge Matias Caviglia, ICRC, Room 917A, New York, NY 10032; phone: 212-851-5464; jmc2245@cumc.columbia.edu.

Keywords

Fibrosis; hepatocellular carcinoma; cholangiocarcinoma; tumor; miR; micro RNA

Hepatic stellate cells (HSC) are key contributors to liver fibrosis (1–3). In the context of liver injury, HSC differentiate from lipid-storing pericytes to extracellular matrix-producing myofibroblasts. A number of pathways, including TGFβ and PDGF signaling, have been identified as important contributors to the myofibroblastic phenotype of HSC in the injured liver (2,4). In the recent decade, microRNAs have emerged as key regulators of gene expression and several studies have suggested a role for microRNAs in regulating HSC activation, making them desirable targets for anti-fibrotic therapies (5–7).

MicroRNAs regulate gene expression by a combination of translational repression and mRNA destabilization (8). They constitute about 1–2% of all genes in mammals, and more than 60% of protein-coding genes are predicted to contain microRNA target sequences (9). Accordingly, microRNAs have been implicated in a wide range of physiological as well as pathological processes (10,11). On the other hand, microRNAs exert only moderate effects on the levels of protein expression, typically less than 2-fold (9). Accordingly, many microRNAs can be deleted without producing an overt phenotype, leading to the suggestion that this system modulates or reinforces the stability of biological systems rather than constituting a primary driver (9). One particular microRNA, miR-21, has been shown to contribute to the development of fibrosis in multiple organs, including lung, kidney and heart (12–14). However, the role of miR-21 in fibrogenesis has been controversial, with some studies demonstrating a promotion of fibrosis by miR-21 but others demonstrating no influence (14–17). Targeting of miR-21 has been patented for treatment of fibrosis in several organs, including liver. Moreover, miR-21 expression is upregulated in many tumors and a large body of literature has implicated miR-21 as well as other microRNAs in carcinogenesis, designing them as “oncomirs” (18–20).

In the liver, miR-21 is believed to regulate a wide range of injury responses, including hepatocyte proliferation (21), biliary hyperplasia (22), carcinogenesis (23) and liver fibrosis (22–25). Here, we identified miR-21 as the microRNA with the highest induction in HSC in multiple models of HSC activation. However, two different lines of miR-21 knockout mice as well as pharmacological suppression of miR-21 expression did not reveal a role for this microRNA in HSC activation or liver fibrosis. Moreover, HSC-specific deletion of Dicer1, an RNAse that is required for the generation of mature microRNAs, had no effect on HSC activation and only minimally affected liver fibrosis, suggesting that microRNAs do not play a crucial role in driving HSC activation or maintaining their activated phenotype. Finally, our data in miR-21 knockout mice and in antimir-treated mice did not reveal a major role for miR-21 in the development of fibrosis-associated liver cancer.

METHODS

Mice

miR-21^{KO} and floxed miR-21 mice were a gift from Dr. Olson (26). Mice expressing Alb-Cre (Jax#003574) and a second line of miR-21^{KO} mice (Jax#016856) (27) were from Jackson. For HSC-specific ablation of Dicer, floxed Dicer1 mice (28) were crossed with LRAT-Cre mice (1). When indicated, mice expressed also the Cre reporter ZsGreen (Jax#007906). MDR2^{KO} mice (1) and mice carrying conditional alleles for PTEN and TGFBR2 have been described (29). Further details are described in the supplements.

Hepatic stellate cell isolation and culture

Mouse HSC were isolated by *in situ* liver perfusion and cultured as described (30–32). Further details are described in the supplements.

Mouse models of liver fibrosis

To induce toxic liver fibrosis, eight week-old mice were administered CCl_4, (0.5 μl/gram body weight, dissolved in corn oil at a ratio 1:3) every three days, for a total of 8 doses, given by intraperitoneal injection or gavage, as indicated. Mice were euthanized 48 hours after the last dose. For cholestatic liver fibrosis, eight week-old mice were subjected to ligation of the common bile duct as described (31). As additional models of cholestatic liver fibrosis, we used MDR2^{KO} mice, or mice treated with diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for three weeks. While we used male mice for most experiments, some experiments were repeated with female mice, including CCl_4-induced fibrosis and MDR2^{KO}-induced liver fibrosis as indicated.

Mouse models of liver cancer

Hepatocellular carcinoma (HCC) was induced by a single dose of diethylnitrosamine (DEN, 25 μg/g, i.p.), administered to male mice at day 15 postpartum, followed by 10 injections with CCl_4 (0.5 μl/g, i.p., once weekly), starting at week 6. In a second model, six week-old male C3H/HOJ mice were treated with DEN (100 μg/g, i.p.), followed by 22 injections of CCl_4 (0.5 μl/g, i.p.). Mice with Alb-Cre-mediated deletion of *Pten* and *Tgfbr2* were used as model of cholangiocarcinoma (29), using a floxed miR-21 allele to simultaneously delete miR-21 in cells from which cholangiocarcinomas originate. In a second model, cholangiocarcinoma was induced by hydrodynamic tail vein injection of plasmids encoding sleeping beauty, pT3-myrAKT and pT3-YapS127A as described (33).

Additional methods are described in the supplements.

RESULTS

miR-21 increases in activated HSC and in the fibrotic liver

Following liver injury, HSC activate and represent the main cell type responsible for the development of liver fibrosis (1,2). To identify microRNAs that may regulate HSC activation in the fibrotic liver, we performed a microarray-based screen of microRNA expression. For this screen, we compared freshly-isolated, never-plated HSC from mice with toxic fibrosis

induced by CCl_4 treatment or biliary liver injury induced by bile duct ligation, to quiescent HSC. In addition to these two *in vivo* models of HSC activation, we also included HSC that were culture-activated in plastic dishes (Suppl. Fig. 1A;B) (32). Although each of these three models of HSC activation resulted in specific alterations of microRNA expression, there was considerable overlap between these models (Fig. 1A–B). Twenty-two microRNAs showed significant changes that were common to all three models, thus representing a microRNA signature of HSC activation (Fig. 1A–B, Table 1). Among those microRNAs, miR-21, a microRNA with known roles in pulmonary, renal and, potentially, cardiac fibrosis (12–15), showed the greatest change, increasing 8–24 fold as determined by microarray and confirmatory qPCR (Fig. 1B–C). Moreover, miR-21 was one of four microRNAs that was significantly upregulated (Fig. 1B), thus making it amenable to antimir-based therapeutic approaches. To confirm these findings, we also isolated HSC from MDR2 *null* ($MDR2^{KO}$) mice, and mice treated with DDC as two additional models of liver fibrosis. Indeed, miR-21 was increased to a similar extent in both models (Fig. 1C). To determine whether the upregulation of miR-21 was due to increases in transcription, we measured its precursor *primir-21. Primir-21* was increased in all *in vivo* models of fibrosis (Fig. 1D). In contrast, culture activation only resulted in a transient increase in *primir-21* expression, followed by a decrease after 5 days, despite a pronounced increase in miR-21 levels (Fig. 1D; Suppl. Fig. 1C). miR-21b was not detectable, while miR-21c was not changed in any of the employed HSC activation models (Suppl. Fig. 1D). Taken together, these results indicate that the induction in miR-21 expression is a characteristic change during HSC activation and that miR-21 may represent an anti-fibrotic target.

miR-21 is not required for the development of toxic and biliary liver fibrosis

Next, we used well-established models of toxic and biliary liver fibrosis to test the role of miR-21in liver fibrogenesis. Following treatment of miR-21 *null* ($miR\text{-}21^{KO}$) (15) and wild-type (WT) controls with eight injections of CCl_4, we did not observe significant differences in Sirius red staining (Fig. 2A) and hepatic hydroxyproline content (Fig. 2B). Moreover, markers of HSC activation, including α-SMA protein and of *Acta2*, *Col1a1*, *Lox,* and *Timp1* mRNA, increased in CCl_4-treated livers but were similar between $miR\text{-}21^{KO}$ mice and WT controls (Fig. 2C–D). mRNA and protein expression of HSC marker desmin increased in livers from CCl_4-treated mice, indicating that HSC expanded to similar degree in $miR\text{-}21^{KO}$ and WT mice during fibrogenesis (Fig. 2D and Suppl. Fig. 6D). There were also no differences in liver injury, as determined by plasma ALT levels, or inflammation as assessed by *Tnf* and *Ccl2* mRNA expression (Fig. 2E–F). In addition to $miR\text{-}21^{KO}$ mice, we also employed inhibitory antisense DNA oligonucleotides (miR-21KD antimirs) to acutely inhibit miR-21 immediately before and during the induction of fibrosis by CCl_4 and address the potential concern that absence of miR-21 throughout development and adulthood could lead to compensatory changes (16). Treatment of mice with miR-21KD antimirs decreased miR-21 levels by 94% and 85% in non-fibrotic and fibrotic livers, respectively, when compared to mice treated with scrambled control oligonucleotides (Suppl. Fig. 2A–B) In addition, the expression of miR-21 target gene *Timp3* (34) was de-repressed in non-fibrotic control livers, but not in fibrotic livers, in which other regulatory mechanisms may predominate (Suppl. Fig. 2B). To determine whether our antimir strategy decreased miR-21 in HSC, we isolated HSC from mice treated with miR-21KD antimir or scrambled control

oligos. qPCR demonstrated an 80% decrease of miR-21 levels in HSC from miR-21KD-treated mice (Suppl. Fig. 2C), thus confirming efficient knockdown in this cell type. However, similar to the results in mirR-21^{KO} mice, acute inhibition of miR-21 by miR-21KD antimirs did not reduce the development of liver fibrosis, as determined by Sirius red staining and hepatic hydroxyproline measurement (Suppl. Fig. 2D–E), or the mRNA expression of HSC activation markers *Acta2*, *Col1a1*, and *Timp1* in whole liver (Suppl. Fig. 2F). Moreover, miR-21KD antimirs did not reduce proliferation, as determined by qPCR for *Mki67* mRNA (Suppl. Fig. 2F). To address the possibility that the dose of miR-21KD might be insufficient, or that the eight doses of CCl_4 may induce pronounced fibrosis that may mask subtle changes, we conducted an additional study: Mice received a high dose of miR-21KD (25 mg/kg), resulting in complete suppression of hepatic miR-21 expression compared to scrambled control oligonucleotides, followed by four doses of CCl_4. Although collagen deposition was evident in this briefer protocol, it was less pronounced. Importantly, there were no differences in Sirius Red staining, and *Acta2*, *Col1a1*, and *Timp1* liver mRNA expression (data not shown), thus excluding that the absent effect on HSC activation and fibrosis in our previous experiments was caused by incomplete suppression of miR-21.

We next evaluated the role of miR-21 in MDR2^{KO} mice and mice fed DDC diet, two well-established models of biliary liver fibrosis. As expected, MDR2^{KO} mice displayed robust liver fibrosis. However, in MDR2^{KO}/miR-21^{KO} double knockout mice, fibrosis development did not differ from that in MDR2^{KO} single knockout mice, as evidenced by similar deposition of fibrillar collagen and similar hepatic hydroxyproline content (Fig. 3A). Likewise, the hepatic expression of fibrogenic genes *Col1a1, Lox,* and *Timp1* as well as HSC marker *Des* was comparable between MDR2^{KO} mice that were wild-type or knockout for miR-21 (Fig. 3C). The expression of *Acta2* mRNA did not increase in this model of biliary fibrosis (Fig. 3C), consistent with previous studies from our lab. In addition, liver injury was not different between miR-21^{KO} and WT mice, as measured by plasma ALT levels (Fig 3B). Similarly to our findings in miR-21^{KO} mice, treatment with miR-21KD antimirs did not significantly alter liver fibrosis or markers of HSC activation in MDR2^{KO} mice, as assessed by Sirius red staining, hepatic hydroxyproline measurement, and qPCR for *Acta2, Col1a1* and *Timp1* mRNA in livers, either in male (Suppl. Fig. 3 A–C) or in female (Suppl. Fig. 3D–F) mice. We additionally investigated the effect of miR-21 deletion in biliary fibrosis induced by DDC. miR-21^{KO} mice fed a diet containing 0.1% DDC displayed no significant differences in liver fibrosis and markers of HSC activation and proliferation in comparison with WT mice, as demonstrated by Sirius red staining, hepatic hydroxyproline content, and *Acta2*, *Col1a1*, *Lox, Timp1,* and *Des* mRNA expression in livers (Fig. 3D–F). Similar to our findings in miR-21^{KO} mice, treatment with miR-21KD antimir did not prevent the development of DDC-induced liver fibrosis (Suppl. Fig. 4A–C). To further confirm these findings, we investigated the effect of miR-21 in BDL-induced fibrosis. Treatment with miR-21KD antimir did not significantly inhibit BDL-induced liver fibrosis or injury, as assessed by Sirius red staining, hepatic hydroxyproline content, hepatic fibrogenic gene mRNA expression, and ALT levels (Suppl. Fig. 5A–D).

As a third approach, in addition to miR-21^{KO} mice (15) and miR-21KD antimir, we determined the effect of miR-21 deletion on liver fibrosis in a second miR-21^{KO} mouse line (27). Again, we did not observe a role for miR-21 in liver fibrosis and HSC activation

induced by CCl_4 in this second miR-21^{KO} mouse line (Suppl. Fig. 6). In summary, our data from three different experimental approaches in a wide range of fibrosis models indicate that miR-21 is not essential for the development of murine liver fibrosis.

miR-21 is not required for culture-activation of HSC

Next, we investigated whether miR-21 was necessary for HSC activation in cell culture as this model resulted in the strongest upregulation of miR-21. Treatment of HSC with miR-21KD antimirs decreased miR-21 in cultured HSC in a dose-dependent manner (up to 99% at the highest dose) in comparison to scrambled control oligonucleotides, without affecting its precursor primir-21 (Fig. 4A). However, miR21-KD antimir treatment had no effect on HSC activation, as determined by expression of α–SMA protein and mRNA expression of fibrogenic genes *Acta2*, *Col1a1*, and *Timp1* (Fig. 4B–C).

miR-21 is not required for the activation of human HSC in culture or in liver slices

To exclude the possibility that miR-21 exerts profibrotic effects in human HSC that cannot be shown in murine models, we evaluated the effect of miR-21KD antimir in primary human HSC. Treatment of human HSC with miR-21KD antimir decreased miR-21 in a dose-dependent manner, achieving up to 99% reduction of miR-21 expression (Fig. 5A). However, similarly to our data in murine HSC, miR-21 knockdown did not affect HSC activation status as determined by α-SMA protein expression and qPCR for *ACTA2*, *COL1A1*, and *TIMP1* mRNA (Fig. 5B–C). As HSCs activated in culture do not fully resemble HSCs activated in vivo (32,35), we additionally evaluated how miR-21 knockdown affects the activation of HSC in liver slices, a model of fibrogenesis in which HSC activate within the liver parenchyma without exposure to artificial cell culture surfaces (36). Despite efficient miR-21 reduction by miR-21KD antimir (Fig. 5D), we again did not observe an effect on HSC activation as determined by qPCR for *ACTA2*, *COL1A1*, and *TIMP1* mRNA (Fig. 5E).

MicroRNA reduction by HSC-specific deletion of Dicer does not significantly alter HSC activation or liver fibrosis

MicroRNAs are generated in a multistep process with a key role for Dicer1 (37). Dicer1 deletion blocks the generation of microRNAs and causes important alterations in organ development and function (38). In particular, Dicer deletion decreases miR-21 and other microRNAs such as let-7 (28). To further evaluate the importance of microRNAs in HSC activation and liver fibrosis, we generated mice with a HSC-specific deletion of Dicer1 (Dicer$^{\Delta HSC}$) by crossing Dicer$^{f/f}$ mice (28) with Lrat-Cre mice (1). This approach resulted in efficient deletion of Dicer1 in HSC with a 94% decrease in *Dicer1* mRNA and decreases in the levels of miR-21, miR-199a-3p and let-7i of at least 50% (Fig. 6A, Suppl. Fig. 7A). To determine the role of microRNA modulation by Dicer1 deletion, we first needed to exclude that Dicer1 deletion resulted in spontaneous activation of HSC or that it severely disturbed the phenotype of HSC. For this purpose, we isolated HSCs from Dicer$^{\Delta HSC}$ and Dicer$^{+/+}$ Lrat-Cre control mice. HSC isolated from Dicer$^{\Delta HSC}$ mice had a normal phenotype with presence of characteristic retinoid-containing lipid droplets (Suppl. Fig. 7B, Day1, left panels). Moreover, RNA sequencing and qPCR showed no alterations in a panel of characteristic HSC genes including *Hand2*, *Lhx2* and *Lrat,* or in fibrosis markers *Acta2,*

Col1a1, and *Timp1*, with the exception of *Lox*, which was upregulated in HSC from Dicer$^{\Delta HSC}$ mice in our RNA seq data but did not show statistical significance in the qPCR (Fig. 6A, Suppl. Fig. 7C, Suppl. Table 1). Moreover, there was no change in the expression of *Pdgfrb*, *Tgfbr2* (Suppl. Fig. 7C). When cultured to induce activation, HSC isolated from Dicer$^{\Delta HSC}$ mice activated similarly to wild-type control cells, showing the characteristic fibroblastic phenotype with partial loss of retinoids (Suppl. Fig. 7B, Day 5, right panels) and similar expression of α-SMA (Suppl. Fig. 7D). To evaluate the effect of Dicer1 deletion in HSC on liver fibrosis, we treated Dicer$^{\Delta HSC}$ mice and control mice with CCl_4. There was no difference in liver injury, as assessed by plasma ALT activity (Fig. 6B). We detected a small but statistically significant reduction in some but not all fibrosis markers in Dicer$^{\Delta HSC}$ mice. Dicer$^{\Delta HSC}$ livers had modestly decreased Sirius red staining, but there was not difference in hepatic hydroxyproline content (Fig. 6C). Likewise, Dicer$^{\Delta HSC}$ livers displayed a decrease in *Col1a1* mRNA expression, but no differences in the expression of other fibrogenesic genes including *Acta2*, *Lox* or *Timp1* (Fig. 6D). However, as Dicer$^{\Delta HSC}$ mice were significantly smaller than their Dicer$^{f/f}$ control mice (Suppl. Fig. 7F) – most likely due to extrahepatic deletion of *Dicer1* related to the high expression of *Lrat-Cre* in several organs during development – it cannot be excluded that the observed minor role of Dicer in HSC activation and liver fibrosis was either exaggerated or masked by differences in body weight. To further investigate the effect of Dicer deletion in the absence of such a confounder, we deleted *Dicer1* in HSC *in vitro* and studied the effect on culture-activation. For this purpose, we isolated HSC from Dicer$^{f/f}$ mice, followed by transduction with an adenovirus encoding Cre recombinase (Ad5-CMVCre) or an empty control virus (Ad5-CMVempty), and evaluation of HSC activation status after 7 days of culture. Cre-transduced cells showed a 98% decrease in *Dicer1* mRNA with associated decreases in microRNAs miR-21 and let-7i in comparison with control adenovirus-infected HSC (Fig. 6E). Moreover, *Dicer1* mRNA was already 98% reduced 72h hours after infection with Ad5-CMVCre, demonstrating that reduced *Dicer1* expression was present for most of the culture activation period in these experiments. However, Dicer1 deletion had minimal effects on HSC activation with no changes in α-SMA protein expression, similar levels of *Acta2*, *Lox* and *Timp1*, and only a moderate increase in *Col1a1* mRNA (Fig. 6E–F). Likewise, we found no alterations in phenotype and α-SMA protein expression in HSC from Dicer$^{\Delta HSC}$ mice that were culture-activated for 5 days and displayed a significant decrease in *Dicer1* expression (Suppl. Fig. 7D,E), thus further excluding that the possible presence of Dicer during the first 72 hours of culture activation in our adenoviral deletion experiments could be responsible for the negative results. Although Dicer deletion may represent a broad and unselective method that reduces the levels of all microRNAs, i.e. those that increase as well as those that decrease during HSC activation, our *in vitro* and *in vivo* data suggest that Dicer and overall microRNA levels do not exert a major role in the regulation of HSC activation, and that the HSC activation program is neither profoundly inhibited nor accelerated in the absence of Dicer and the associated decrease of microRNA levels.

miR-21 is not required for hepatocarcinogenesis

We next investigated the hypothesis that miR-21 might contribute to the development of liver cancer. Of note, liver cancer is commonly associated with chronic liver injury and fibrosis (39) and represents the main cause of mortality in patients with compensated liver

cirrhosis (40). Upregulation of miR-21 has been reported in a large number of malignancies, including HCC and cholangiocarcinoma (41–43) and miR-21 has been termed an “oncomir” based on functional studies (20). Similarly to previous studies (41,42), we observed increased miR-21 expression in liver cancer, including HCC induced by DEN+CCl_4, HCC induced by liver parenchymal cell (LPC)-specific *Pten* deficiency and cholangiocarcinoma induced by LPC-specific deletion of *Pten* and *Tgfbr2* (Fig. 7A). To evaluate the contribution of miR-21 to liver carcinogenesis, we induced HCC in miR-21^{KO} and WT mice by the combination of DEN and CCl_4 (Fig. 7B). There were no differences in tumor development between miR-21^{KO} and WT mice, with similar liver-to-body weight ratio, tumor number, and size in both groups (Fig. 7C). In a complementary approach, we determined effects of miR-21KD antimirs on DEN+CCl_4-induced HCC. To allow for efficient miR-21 knockdown, we chose an adult tumor model in which C3H mice are treated with DEN at the age of 6 weeks, followed by 22 weekly doses of CCl_4 (Fig. 7E) (44). Tumors showed increased expression of HCC marker Gpc3 and proliferation marker Mki67 (Suppl. Fig. 8A). Treatment with miR-21KD antimir efficiently decreased miR-21 in tumors and surrounding non-tumor tissue (Fig. 7D). Consistent with our studies in knockout mice, miR-21 knockdown did not alter tumor development (Fig. 7E). Moreover, analysis of gene expression within tumors showed no differences in genes that have been reported to be involved in carcinogenesis and are predicted to be regulated by miR-21 (Suppl. Fig. 8B).

Next, we evaluated whether miR-21 affects carcinogenesis in two cholangiocarcinoma models. First, we used a model of CCA driven by deletion of PTEN and TGFBR2 (29). In this model, co- deletion of miR-21 increased the tumor number and liver-to-body weight ratio but not tumor size (Fig. 7F). Second, we used a CCA model driven by overexpression of activated AKT and Yap (33) and employed either miR-21^{KO} mice or miR-21KD antimirs. By both approaches, we did not observe differences in cholangiocarcinoma development, as determined by liver-to-body weight ratio, the keratin 19-positive area and *Krt19* and *Prom1* mRNA expression (Suppl. Fig. 9). These results are in agreement with the lack of any effect of miR-21KD on the hepatic expression of cancer-related target genes (Suppl. Fig. 10).

To further determine the role of miR-21 in human liver cancer, we studied four liver cancer cell lines as well as two non-transformed controls in vitro. In HepG2 and PLC/PRF/5, transfection with miR-21KD decreased miR-21 levels 97% and 99%. However, it did not affect proliferation, apoptosis, or anchorage-independent growth (Suppl. Fig. 11). Similarly, miR-21KD decreased miR-21 in HuCCT-1 and KMCH cells but did not affect those cancer properties (Suppl. Fig. 12). In addition, transfection of hepatocyte cells THLE-2 and cholangiocyte cells H69 with miR-21 increased miR-21 levels, but it did not affect proliferation, apoptosis, and anchorage independent growth (Suppl. Fig. 13). These cell culture data suggest that miR-21 is not essential for several hallmark properties, nor does it, by itself confer a transformed phenotype to non-cancer cells.

DISCUSSION

Currently, there are no approved anti-fibrotic drugs for the treatment of liver fibrosis. Recent studies on lung, kidney, heart as well as liver fibrosis have reported that blocking miR-21 exerts anti-fibrogenic effects (12–14,22–25) and that miR-21 may therefore represent a

potential therapeutic target. However, studies using both genetic knockout as well as pharmacologic approaches have refuted some of these findings (15,45,46), thus rendering the contribution of miR-21 to fibrosis and its potential use as therapeutic target controversial (16,17). Here, we employed a comprehensive approach, in which we used two different knockout models of miR-21 and pharmacologic antagonists in a wide range of *in vitro* and *in vivo* models of HSC activation and liver fibrosis. Despite a consistent increase in miR-21 in HSC in all investigated models, we found no evidence supporting the hypothesis that miR-21 is essential for the development of liver fibrosis or for HSC activation. Although we cannot completely exclude that miR-21 may have an effect in other fibrosis models, we did not observe changes in HSC activation and liver fibrosis in toxic fibrosis induced by CCl_4, biliary fibrosis induced by $MDR2^{KO}$, DDC diet, or BDL as well as HSC culture activation. Moreover, we excluded that the lack of effect may have been species-specific, as evidenced by unaltered activation of primary human HSC in cell culture and in human liver slices after pharmacologic miR-21 knockdown. The lacking effect of antimir treatment was not caused by insufficient miR-21 knockdown as we achieved >95% inhibition of miR-21 in most experiments. Furthermore, we excluded that this decrease was an artifact due to residual miR-21-KD that may have been co-extracted with the RNA and interfered with the RT or qPCR assays (Suppl. Fig. 14). Despite efficient knockdown of miR-21, we only observed a moderate increase in the expression of recognized miR-21 target *Timp3* mRNA (Suppl. Fig. 2B) (14,26,34), and a trend towards increased SPRY1 expression in some but not other experiments (data not shown). These variable and mostly absent effects are consistent with our finding of unaltered PDCD4 and SPRY1 protein in livers of $miR\text{-}21^{KO}$ mice (data not shown) in our studies as well as previous studies in hearts of $miR\text{-}21^{KO}$ mice (15). These findings support the notion that miR-21 exerts little influence on gene expression in the liver as previously shown by others (34), whereas its role in other organs such as the skin may be more potent as evidenced by moderately upregulated target gene expression in keratinocytes of the $miR\text{-}21^{KO}$ mouse (27), employed in our study. Altogether, findings from our and previously published studies are consistent with the concept that the microRNAs confer robustness to biological systems rather than acting as primary mechanism to control expression (9). While genetic knockouts are the cleanest strategy to study the role of miR-21 in fibrosis and cancer, it has been suggested by some (16) but refuted by others (15) that miR-21 knockout may trigger compensatory mechanisms. However, we did not find compensatory upregulation of miR-21b or miR-21c in either of the two $miR\text{-}21^{KO}$ models (Suppl. Fig. 14B–C). Moreover, compensation appears unlikely for the miR-21 knockout mouse as it is resistant to lung tumor development (26) and as other miR knockout mice do not show compensation (47). Our results differ from two previous publications, which have reported that miR-21 promotes liver fibrosis (23,24). Some of these discrepancies may be due to the use of different fibrosis models or due to different antimirs. Antimirs of different lengths have been reported to ameliorate or not affect cardiac fibrosis (14–16). Our studies in two independently generated miR-21 knockout mice as well as pharmacologic knockdown in a total of four *in vivo* fibrosis models, cultured human HSC and human liver slices all yielded similar data, supporting the conclusion that targeting miR-21 may not be a promising anti-fibrotic therapy and that other microRNAs, e.g. miR-29, may provide better targets (48).

To further understand the overall role of microRNAs in HSC biology and liver fibrosis, we deleted *Dicer1* in HSC *in vitro* and *in vivo*. By both approaches, we found a significant downregulation of microRNA expression in HSC, but largely unaltered HSC activation and fibrosis with the majority of readouts unaltered. Although the observed alteration in body weight in Dicer$^{\Delta HSC}$ mice makes it difficult to exclude a role for HSC in the fibrogenic process, we did not observe an inhibition of HSC activation by Dicer deletion in the culture activation model either. Our finding that a number of genes were altered in Dicer-deleted HSC suggests that microRNAs modulate the expression of specific genes in HSC, but that those genes are not involved in the programs that control the identity and activation of HSC. This is consistent with the finding that microRNAs only exert a minor effect on protein expression in many systems and that their purpose is to stabilize rather than drive biological processes (9). As Dicer deletion represents a broad and non-specific method, we cannot exclude that our results are due to concomitant reduction of microRNAs that promote HSC activation and microRNAs that antagonize HSC activation, and that this resulted in the absence of a net effect. This is further supported by the finding that miR-29 contributes to HSC activation and liver fibrosis (48).

The development of liver cancer represents another key consequence of chronic liver injury and is a leading cause of death in patients with advanced chronic liver disease (40,49). Based on previous reports showing an upregulation of miR-21 in a wide range of tumors including HCC and CCA (41–43) and a potential role as “oncomir” (18–20), we additionally investigated its role in different liver cancer models, again using genetic knockout and antimirs as complementary approaches in our *in vivo* models, as well as cell culture models. Despite an upregulation of miR-21, we did not observe an effect of either miR-21 knockout or antimir treatment on the development of HCC or CCA. Contrasting with our results, Zhang *et al.* have reported that miR-21 promotes PTEN-deletion induced HCC (23); the differences between these data could be due to the use of different HCC models, or to the use of antagonist oligonucleotides, as stated above. One potential weakness in our study is the use of tumor models that are driven by PTEN, a target of miR-21 (42) or its downstram target Akt, which might not allow to reveal PTEN-mediated effects of miR-21 inhibition. However, previous studies also employed miR-21 antagonism in models of PTEN-driven cancer (23), suggesting the presence of PTEN is not absolutely required for potential anti-tumorigenic effects. Although our data indicate no role for miR-21 in liver carcinogenesis, it may have an important role in the response to treatment including chemotherapy. In summary, our results suggest that miR-21 neither affects fibrosis nor fibrosis-associated liver cancer formation and that there should be focus on other microRNA targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support

This study was supported by NIH grants R01CA190844, R01CA200597 and U54CA163111 (to RFS), K22CA178098 and R03DK101863 (to JMC), National Natural Science Foundation of China grant NSFC81300346

(to JY) and an AGA Research Scholar Award (to SA). These studies used the resources of the Cancer Center Flow Core Facility funded in part through Center Grant P30CA013696.

We thank Dr. Eric Olson (UT Southwestern) for miR-21KO and miR-21$^{fl/fl}$ mice, Dr. Dianne Dapito (ETH Zurich), Dr. Jean-Philippe Pradere (INSERM Toulouse), and Dr. Ingmar Mederacke (Medical School Hannover) for help with HSC isolations and BDL, and Dr. Xioyun Sun (Columbia University) for RNAseq data analysis. We thank Deqi Yin, Ana Dilone, and Miguel Rivas (Columbia University) for excellent and humane care of the animals.

Abbreviations

BDL	bile duct ligation
BW	body weight
CCl_4	carbon tetrachloride
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DEN	diethylnitrosamine
HSC	hepatic stellate cells

References

1. Mederacke I, Hsu CC, Troeger JS, Huebener P, Mu X, Dapito DH, Pradere JP, et al. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. Nat Commun. 2013; 4:2823. [PubMed: 24264436]
2. Puche JE, Saiman Y, Friedman SL. Hepatic stellate cells and liver fibrosis. Compr Physiol. 2013; 3:1473–1492. [PubMed: 24265236]
3. Wells RG, Schwabe RF. Origin and function of myofibroblasts in the liver. Semin Liver Dis. 2015; 35:97–106. [PubMed: 25974896]
4. Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. Annu Rev Pathol. 2011; 6:425–456. [PubMed: 21073339]
5. Lambrecht J, Mannaerts I, van Grunsven LA. The role of miRNAs in stress-responsive hepatic stellate cells during liver fibrosis. Front Physiol. 2015; 6:209. [PubMed: 26283969]
6. Szabo G, Bala S. MicroRNAs in liver disease. Nat Rev Gastroenterol Hepatol. 2013; 10:542–552. [PubMed: 23689081]
7. Wang XW, Heegaard NH, Orum H. MicroRNAs in liver disease. Gastroenterology. 2012; 142:1431–1443. [PubMed: 22504185]
8. Jonas S, Izaurralde E. Towards a molecular understanding of microRNA-mediated gene silencing. Nat Rev Genet. 2015; 16:421–433. [PubMed: 26077373]
9. Ebert MS, Sharp PA. Roles for microRNAs in conferring robustness to biological processes. Cell. 2012; 149:515–524. [PubMed: 22541426]
10. Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. Cell. 2012; 148:1172–1187. [PubMed: 22424228]
11. Beermann J, Piccoli MT, Viereck J, Thum T. Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches. Physiol Rev. 2016; 96:1297–1325. [PubMed: 27535639]
12. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, Kaminski N, et al. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. J Exp Med. 2010; 207:1589–1597. [PubMed: 20643828]
13. Chau BN, Xin C, Hartner J, Ren S, Castano AP, Linn G, Li J, et al. MicroRNA-21 promotes fibrosis of the kidney by silencing metabolic pathways. Sci Transl Med. 2012; 4:121ra118.

14. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature. 2008; 456:980–984. [PubMed: 19043405]
15. Patrick DM, Montgomery RL, Qi X, Obad S, Kauppinen S, Hill JA, van Rooij E, et al. Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice. J Clin Invest. 2010; 120:3912–3916. [PubMed: 20978354]
16. Thum T, Chau N, Bhat B, Gupta SK, Linsley PS, Bauersachs J, Engelhardt S. Comparison of different miR-21 inhibitor chemistries in a cardiac disease model. J Clin Invest. 2011; 121:461–462. author reply 462–463. [PubMed: 21285516]
17. Patrick DM, Kauppinen S, van Rooij E, Olson EN. Response to Thum et al. The Journal of Clinical Investigation. 2011; 121:462–463.
18. Adams BD, Kasinski AL, Slack FJ. Aberrant regulation and function of microRNAs in cancer. Curr Biol. 2014; 24:R762–776. [PubMed: 25137592]
19. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. 2006; 6:259–269. [PubMed: 16557279]
20. Medina PP, Nolde M, Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. Nature. 2010; 467:86–90. [PubMed: 20693987]
21. Ng R, Song G, Roll GR, Frandsen NM, Willenbring H. A microRNA-21 surge facilitates rapid cyclin D1 translation and cell cycle progression in mouse liver regeneration. J Clin Invest. 2012; 122:1097–1108. [PubMed: 22326957]
22. Kennedy LL, Meng F, Venter JK, Zhou T, Karstens WA, Hargrove LA, Wu N, et al. Knockout of microRNA-21 reduces biliary hyperplasia and liver fibrosis in cholestatic bile duct ligated mice. Lab Invest. 2016; 96:1256–1267. [PubMed: 27775690]
23. Zhang J, Jiao J, Cermelli S, Muir K, Jung KH, Zou R, Rashid A, 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]
24. Zhang Z, Zha Y, Hu W, Huang Z, Gao Z, Zang Y, Chen J, et al. The autoregulatory feedback loop of microRNA-21/programmed cell death protein 4/activation protein-1 (MiR-21/PDCD4/AP-1) as a driving force for hepatic fibrosis development. J Biol Chem. 2013; 288:37082–37093. [PubMed: 24196965]
25. Coll M, El Taghdouini A, Perea L, Mannaerts I, Vila-Casadesus M, Blaya D, Rodrigo-Torres D, et al. Integrative miRNA and Gene Expression Profiling Analysis of Human Quiescent Hepatic Stellate Cells. Sci Rep. 2015; 5:11549. [PubMed: 26096707]
26. Hatley ME, Patrick DM, Garcia MR, Richardson JA, Bassel-Duby R, van Rooij E, Olson EN. Modulation of K-Ras-dependent lung tumorigenesis by MicroRNA-21. Cancer Cell. 2010; 18:282–293. [PubMed: 20832755]
27. Ma X, Kumar M, Choudhury SN, Becker Buscaglia LE, Barker JR, Kanakamedala K, Liu MF, et al. Loss of the miR-21 allele elevates the expression of its target genes and reduces tumorigenesis. Proc Natl Acad Sci U S A. 2011; 108:10144–10149. [PubMed: 21646541]
28. Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ. The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. Proc Natl Acad Sci U S A. 2005; 102:10898–10903. [PubMed: 16040801]
29. Mu X, Pradere JP, Affo S, Dapito DH, Friedman R, Lefkovitch JH, Schwabe RF. Epithelial Transforming Growth Factor-beta Signaling Does Not Contribute to Liver Fibrosis but Protects Mice From Cholangiocarcinoma. Gastroenterology. 2016; 150:720–733. [PubMed: 26627606]
30. Mederacke I, Dapito DH, Affo S, Uchinami H, Schwabe RF. High-yield and high-purity isolation of hepatic stellate cells from normal and fibrotic mouse livers. Nat Protoc. 2015; 10:305–315. [PubMed: 25612230]
31. Pradere JP, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, Jang MK, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. Hepatology. 2013; 58:1461–1473. [PubMed: 23553591]
32. De Minicis S, Seki E, Uchinami H, Kluwe J, Zhang Y, Brenner DA, Schwabe RF. Gene expression profiles during hepatic stellate cell activation in culture and in vivo. Gastroenterology. 2007; 132:1937–1946. [PubMed: 17484886]

33. Zhang S, Song X, Cao D, Xu Z, Fan B, Che L, Hu J, et al. Pan-mTOR inhibitor MLN0128 is effective against intrahepatic cholangiocarcinoma induced in mice by AKT and Yap co-expression. J Hepatol. 2017
34. Androsavich JR, Chau BN, Bhat B, Linsley PS, Walter NG. Disease-linked microRNA-21 exhibits drastically reduced mRNA binding and silencing activity in healthy mouse liver. RNA. 2012; 18:1510–1526. [PubMed: 22740638]
35. Sancho-Bru P, Bataller R, Gasull X, Colmenero J, Khurdayan V, Gual A, Nicolas JM, et al. Genomic and functional characterization of stellate cells isolated from human cirrhotic livers. J Hepatol. 2005; 43:272–282. [PubMed: 15964095]
36. de Graaf IA, Olinga P, de Jager MH, Merema MT, de Kanter R, van de Kerkhof EG, Groothuis GM. Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. Nat Protoc. 2010; 5:1540–1551. [PubMed: 20725069]
37. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol. 2009; 10:126–139. [PubMed: 19165215]
38. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001; 409:363–366. [PubMed: 11201747]
39. Affo S, Yu LX, Schwabe RF. The Role of Cancer-Associated Fibroblasts and Fibrosis in Liver Cancer. Annu Rev Pathol. 2017; 12:153–186. [PubMed: 27959632]
40. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology. 2004; 127:S35–50. [PubMed: 15508101]
41. Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, Zucman-Rossi J. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology. 2008; 47:1955–1963. [PubMed: 18433021]
42. 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; 133:647–658. [PubMed: 17681183]
43. Selaru FM, Olaru AV, Kan T, David S, Cheng Y, Mori Y, Yang J, et al. MicroRNA-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3. Hepatology. 2009; 49:1595–1601. [PubMed: 19296468]
44. Dapito DH, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, Caviglia JM, et al. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. Cancer Cell. 2012; 21:504–516. [PubMed: 22516259]
45. Lai JY, Luo J, O'Connor C, Jing X, Nair V, Ju W, Randolph A, et al. MicroRNA-21 in glomerular injury. J Am Soc Nephrol. 2015; 26:805–816. [PubMed: 25145934]
46. Moreira Soares Oliveira B, Durbeej M, Holmberg J. Absence of microRNA-21 does not reduce muscular dystrophy in mouse models of LAMA2-CMD. PLoS One. 2017; 12:e0181950. [PubMed: 28771630]
47. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 2007; 316:575–579. [PubMed: 17379774]
48. Roderburg C, Urban GW, Bettermann K, Vucur M, Zimmermann H, Schmidt S, Janssen J, et al. Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. Hepatology. 2011; 53:209–218. [PubMed: 20890893]
49. Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. Gastroenterology. 2014; 147:765–783. e764. [PubMed: 25046161]

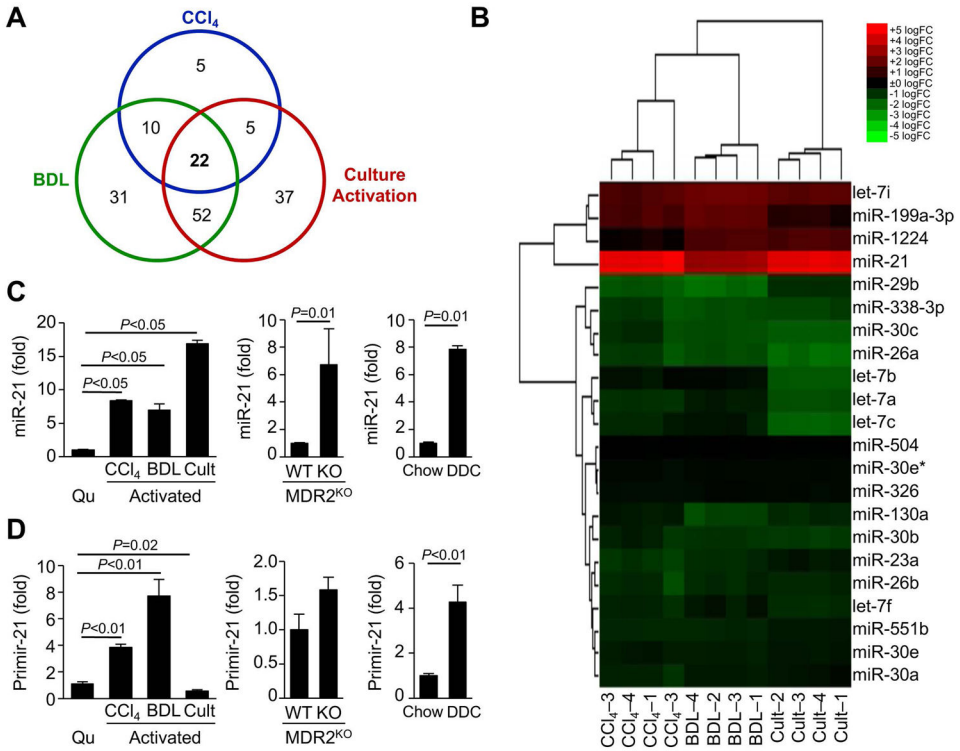

Figure 1. HSC activation leads to changes in microRNA expression

(**A**) Venn diagram of the microRNA that significantly change during CCL_4-, BDL-, and culture-induced HSC activation. (**B**) Heatmap of microRNAs that are altered in all three models of HSC activation. (**C–D**) miR-21 (C) and primir-21 (D) expression were determined by qPCR in CCl_4- BDL- $MDR2^{KO}$-, DDC diet- and culture-activated HSC and are expressed as fold-induction compared to quiescent HSC. HSC were isolated from male mice. N = 4 for A and B; n = 4 to 6 for C and D.

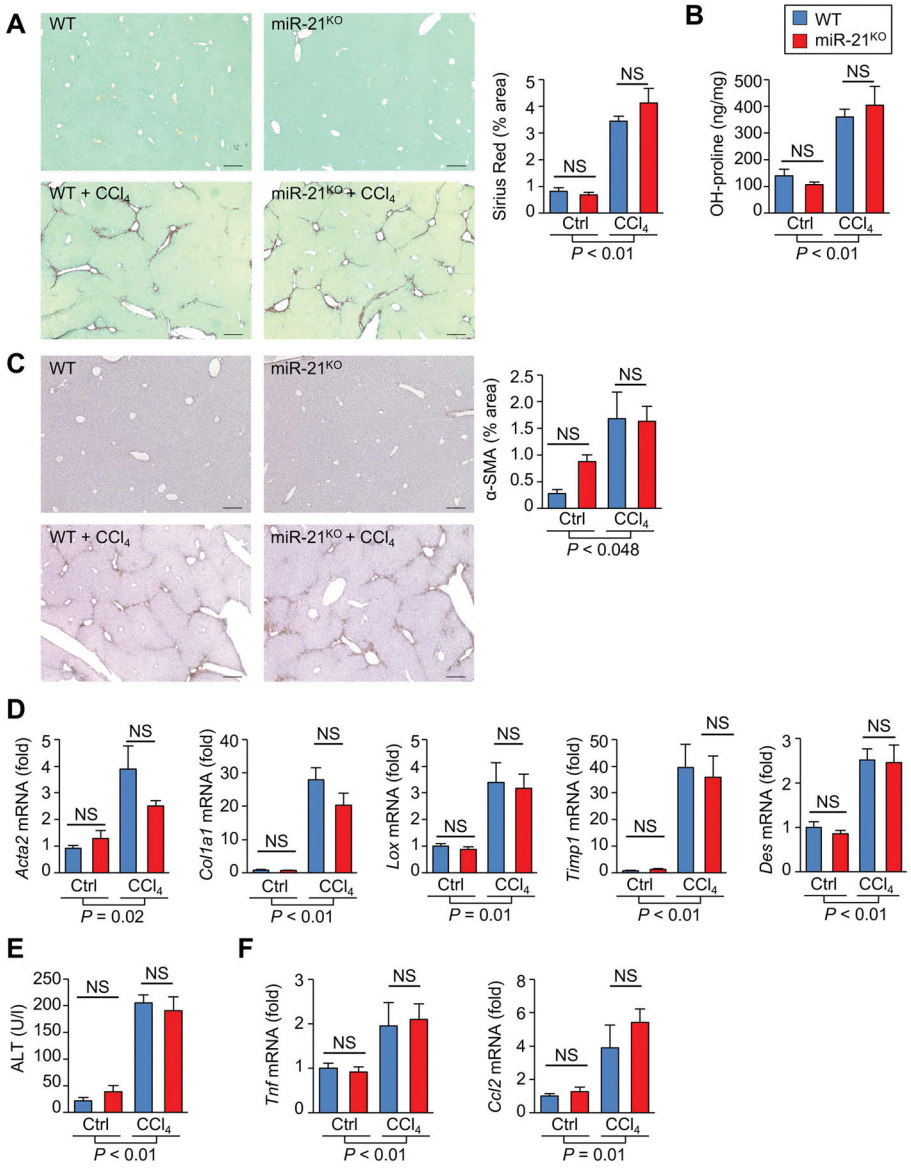

Figure 2. miR-21 deletion does not prevent hepatotoxic fibrosis

WT and miR-21^{KO} female mice (n=9/group) were treated with eight doses of CCl_4; untreated mice were used as control (Ctrl; n=3/group). WT and miR-21^{KO} mice developed similar liver fibrosis as determined by morphometric quantification of the Sirius red-positive area (**A**) or by hepatic hydroxyproline content (**B**). miR-21 deletion did not affect HSC activation as assessed by hepatic α-SMA IHC (**C**) or qPCR for *Acta2*, *Col1a1*, *Lox*, and *Timp1* mRNA or HSC number evaluated by Desmin mRNA expression measured in liver samples (**D**). Hepatocellular injury assessed by plasma ALT activity was similar in WT and KO mice (**E**). Hepatic inflammation was assessed in livers from female WT and miR-21^{KO} mice (n=5 and 6/group) by qPCR for *Tnf*, or *Ccl2* mRNA expression after four CCl_4 injections and did not show differences between WT and KO mice (**F**). Scale bar 200 μm. NS: Not statistically significant.

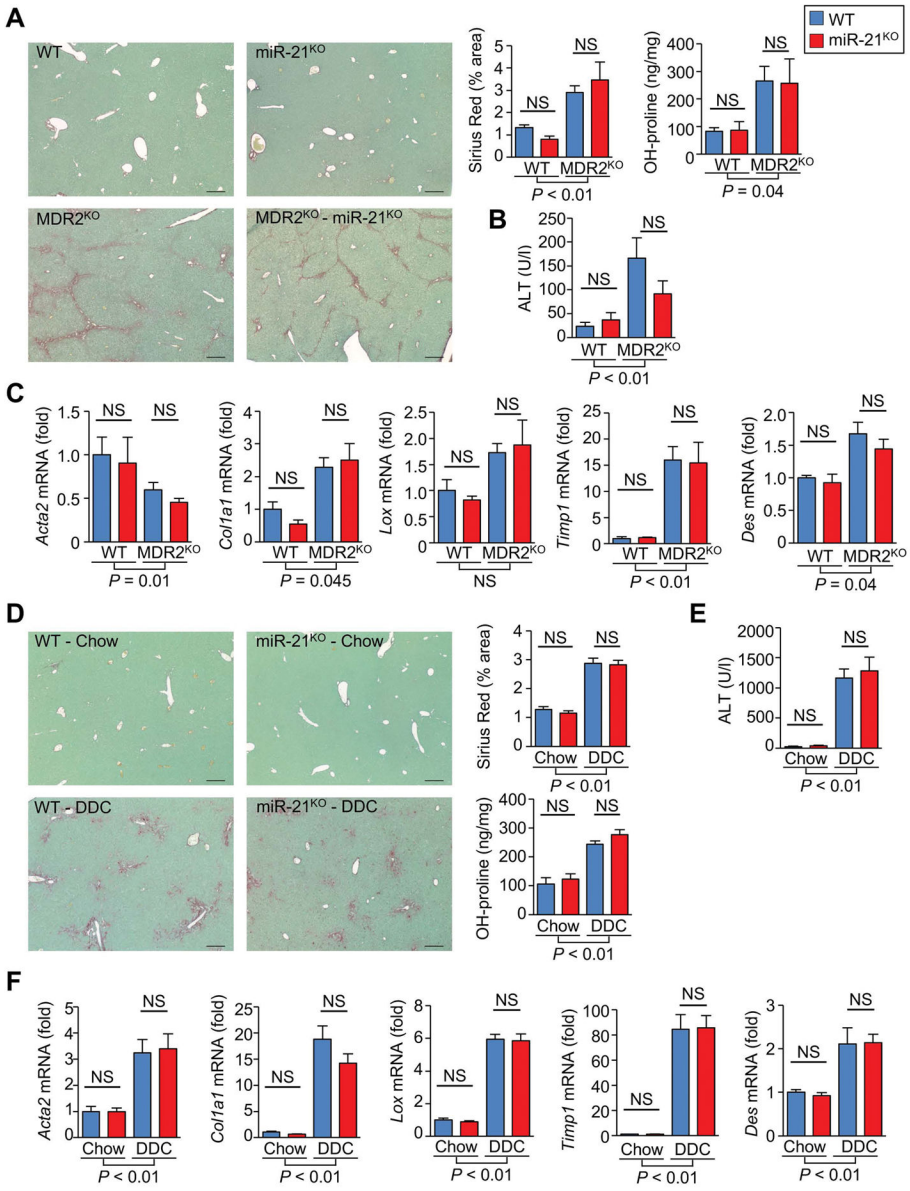

Figure 3. Deletion of miR-21 does not prevent cholestatic liver fibrosis

Genetic deletion of miR-21 in MDR2^{KO} male mice (n=9) did not reduce fibrosis, as determined by Sirius red staining (**A**), hepatic hydroxyproline content (**A**, right panel), and *Acta2, Col1a1, Lox,* and *Timp1* mRNA expression in liver, HSC expansion assessed by *Des* mRNA expression, (**C**) or liver injury (**B**) when compared to MDR2^{KO} male mice that were WT for miR-21 (n=11); mice wild-type for MDR2 were use as controls (n=3/group). Male WT (n=9) and miR-21^{KO} (n=11) mice fed DDC-containing diet showed similar fibrosis, HSC expansion, and liver injury, as determined by Sirius red staining (**D**), hepatic hydroxyproline content (**D**, right panel), qPCR for *Acta2*, *Col1a1, Lox*, *Timp1* and *Des,* in liver (**F)** as well as plasma ALT activity (**E**); mice fed chow were use as controls (n=6 per group). Scale bar 200 μm.

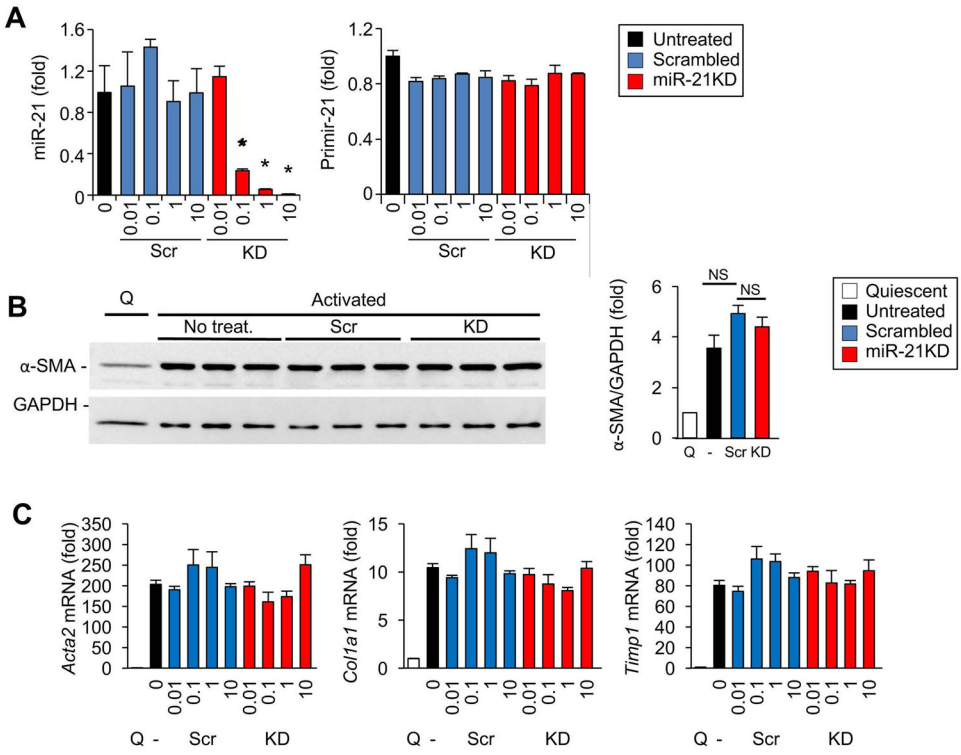

Figure 4. Blocking miR-21 does not prevent mouse HSC activation in culture

Primary murine HSC isolated from male Balb/C mice were plated, allowed to attach overnight, treated with miR-21KD (KD) or scrambled-sequence (Scr) control oligonucleotides at indicated concentrations (in μM) and kept in culture for 5 days to induce activation. miR-21KD treatment decreased miR-21 in a dose-dependent manner, but not Primir-21 (A). Treatment with miR-21-KD, did not affect the activation of HSC, as evaluated by expression of α-SMA determined by western blot (B) or HSC activation markers *Acta2*, *Col1a1*, and *Timp1* mRNA determined by qPCR (C); in B and C, values are expressed as fold-change compared to quiescent HSC. (N=3).

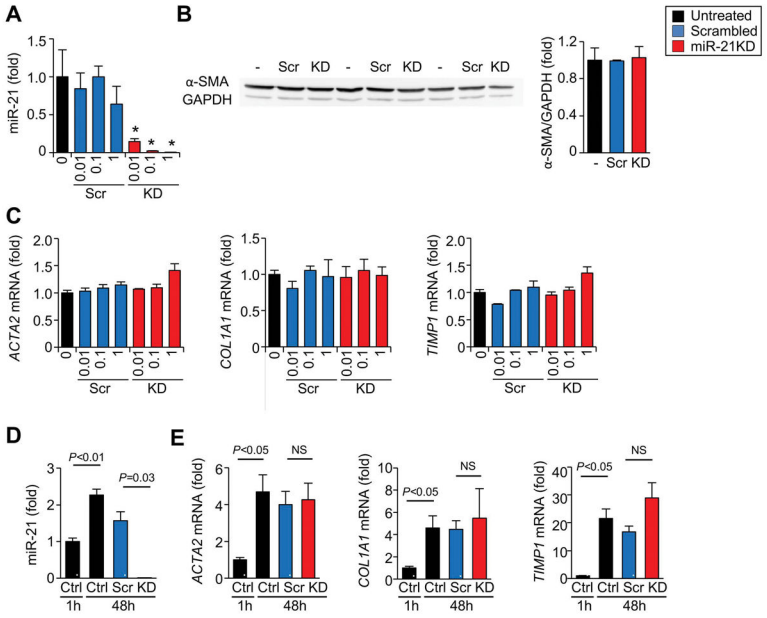

Figure 5. Blocking miR-21 does not prevent human HSC activation

Treatment of human primary HSC with miR-21KD (0.01–1μM) decreased miR-21 levels in a dose-dependent manner in comparison to control oligos (0.01–1μM) (**A**), but did not reduce α-SMA protein expression (1μM) (**B**) or mRNA expression of *ACTA2*, *COL1A1* or *TIMP1* (**C**) (N=3). Treatment of human liver slices with miR-21KD (1μM) decreased miR-21 (**D**) but did not reduce expression of *ACTA2*, *COL1A1*, or *TIMP1* mRNA (**E**) (N=7).

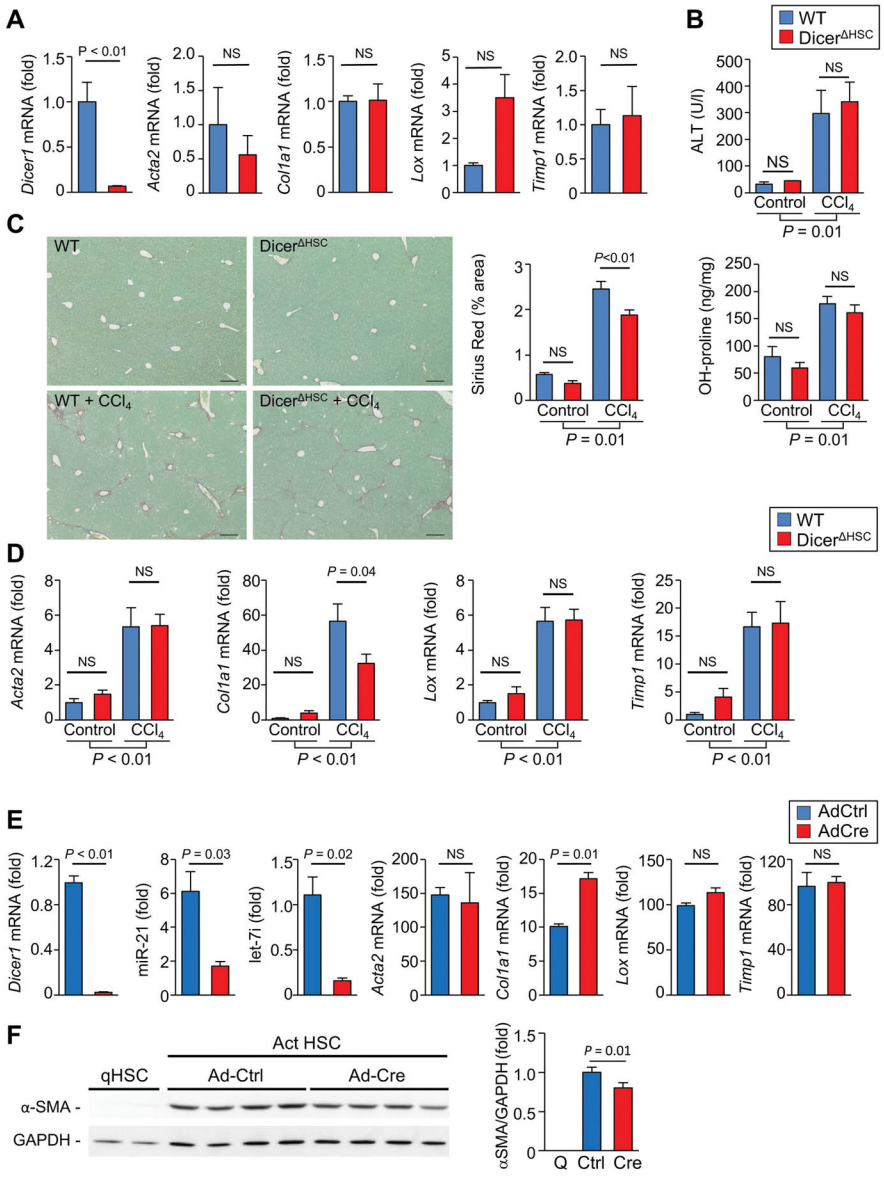

Figure 6. Dicer1 deletion has minimal effects on HSC phenotype, HSC activation and liver fibrosis

(**A**) HSC were isolated from male WT or Dicer$^{\Delta HSC}$ (Δ) mice, and the expression of Dicer1 and HSC activation genes was measured by qPCR in the isolated cells (n=3). (**B–D**) Female WT (n=9) and Dicer$^{\Delta HSC}$ (n=10) mice were treated with four injections of CCl_4. Liver injury was assessed by ALT activity (B), liver fibrosis was assessed by Sirius Red staining (C), hepatic hydroxyproline content (C), and mRNA expression of *Acta2*, *Col1a1*, *Lox* and *Timp1* in whole livers (D). (**E–F**) HSC isolated from Dicer floxed mice were transduced with control (AdCtrl) or a Cre-expressing (AdCre) adenoviruses to delete Dicer, and cultured for 7 days. Dicer deletion was confirmed by QPCR for *Dicer* mRNA and microRNAs miR-21 and let-7i (E). HSC activation was assessed by qPCR for *Acta2*, *Col1a1*, *Lox* and *Timp1* (E) and α-SMA protein expression (F) (n=4/group); values are expressed as fold-change compared to quiescent HSC (Qu). Scale bar 200 μm.

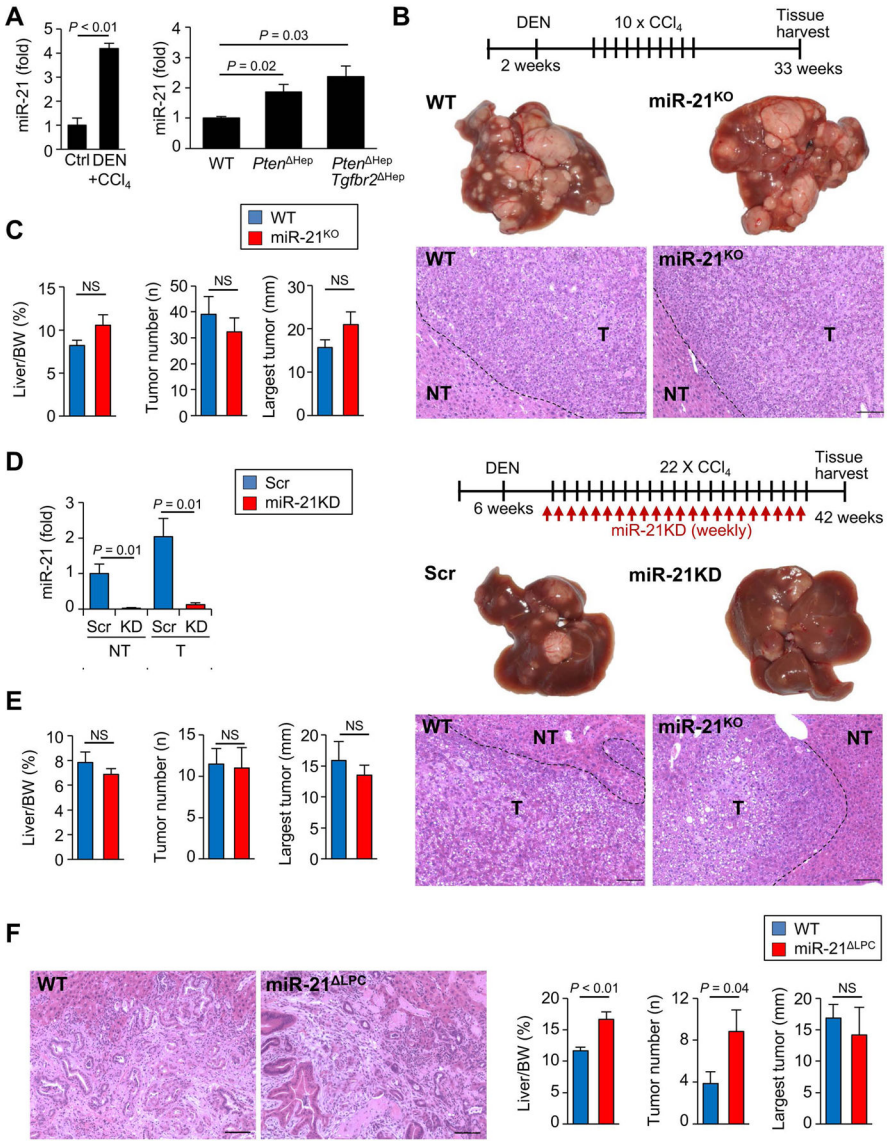

Figure 7. miR-21 does not promote hepatic carcinogenesis

(**A**) miR-21 was determined by qPCR in liver tumors from DEN+CCl_4-treated mice (n=3), from mice with liver-specific deletion of PTEN ($Pten^{\Delta LPC}$, n=4), or PTEN and TGFBR2 (Pten $Tgfbr2^{\Delta LPC}$, n=3), and expressed as fold-induction in comparison to livers from non-treated WT mice (Ctrl, n=3). (**B–C**) Male miR-21^{KO} (n=13) and WT (n=12) mice were treated with DEN+CCl_4 at 2 weeks of age, followed by chronic treatment with CCl_4 to induce liver tumors and tumor development was determined by tumor number, liver-to-body weight ratio, and tumor size. (**D–E**) C3H male mice received DEN+CCl_4 and scrambled oligonucleotides (Scr, n=8) or miR-21-KD (KD, n=8). miR-21-KD decreased miR-21 both in tumors and non-tumor tissue but did not prevent the development of tumors as shown by liver to body weight ratio, tumor number or size. (**F**) LPC-specific deletion of miR-21 (n=6) did not prevent the development of cholangiocarcinoma in Pten $Tgfbr2^{\Delta LPC}$ male mice in

comparison to Pten Tgfbr2$^{\Delta LPC}$ mice with a WT miR-21 allele (n=8), as determined by liver-to-body weight ratio, tumor number and tumor size. T, tumor; NT, non-tumor.