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

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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 microRNA-21 (miR-21), a microRNA that has been implicated in the development of fibrosis in multiple organs and has also been suggested to act as an "oncomir." Accordingly, miR-21 was the microRNA that showed the strongest up-regulation in activated hepatic stellate cells (HSCs) in multiple models of fibrogenesis, with an 8-fold to 24-fold induction compared to quiescent HSCs. However, miR-21 antisense inhibition did not suppress the activation of murine or human HSCs in culture or in liver slices. Moreover, genetic deletion of miR-21 in two independently generated knockout mice or miR-21 antisense inhibition did not alter HSC activation or liver fibrosis in models of toxic and biliary liver injury. Despite a strong up-regulation 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 up-regulated microRNA did not affect HSC activation, liver fibrosis, or fibrosis-associated liver cancer, we additionally tested the role of microRNAs in HSCs by HSC-specific Dicer deletion. Although Dicer deletion decreased microRNA expression in HSCs and altered the expression of select genes, it only exerted negligible effects on HSC activation and liver fibrosis. *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. (HEPATOLOGY 2018;67:2414-2429).

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Hepatic stellate cells (HSCs) are key contributors to liver fibrosis.[1-3] In the context of liver injury, HSCs differentiate from lipid-storing pericytes to extracellular matrix-producing myofibroblasts. A number of pathways, including transforming growth factor beta (TGFβ) and platelet-derived growth factor (PDGF) signaling, have been identified as important contributors to the myofibroblastic phenotype of HSCs in the injured liver.[2,4] During the recent decade,

Abbreviations: α-SMA, α-smooth muscle actin; Acta2, actin 2; ad, adenovirus; Alb-Cre, Cre recombinase under control of Alb promoter/enhancer elements; ALT, alanine aminotransferase; BDL, bile duct ligation; CMV, cytomegalovirus; Col1a1, collagen type I alpha 1; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DEN, diethylnitrosamine; Des, desmin; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; Lox, lysyl oxidase; LPC, liver parenchymal cell; LRAT, lecithin retinol acyltransferase; MDR2, multidrug resistance protein 2; mRNA, messenger RNA; PDGF, platelet-derived growth factor; PTEN, phosphatase and tensin homolog; qPCR, quantitative polymerase chain reaction; TGFBR2, transforming growth factor beta receptor 2; TGFβ, transforming growth factor beta; Timp, tissue inhibitor of metalloproteinase; WT, wild type.

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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 antifibrotic therapies.[5-7]

MicroRNAs regulate gene expression by a combination of translational repression and messenger RNA (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 physiologic as well as pathologic processes.[10,11] On the other hand, microRNAs exert only moderate effects on 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 constitutes a primary driver.[9] One particular microRNA, mature microRNA-21 (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 promotion of fibrosis by miR-21 but others demonstrating no influence.[14-17] Targeting of miR-21 has been patented for the treatment of fibrosis in several organs, including liver. Moreover, miR-21 expression is up-regulated in many tumors, and a large body of literature has implicated miR-21 as well as other microRNAs in carcinogenesis, designating 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 HSCs in multiple models of HSC activation. However, two diferent lines of miR-21 knockout mice as well as pharmacologic 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, a ribonuclease 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 from miR-21 knockout mice and from antimir-treated mice did not reveal a major role for miR-21 in the development of fibrosis-associated liver cancer.

Materials and Methods

MICE

miR-21 null (miR-21^{KO}) and floxed miR-21 mice were a gift from Dr. Olson (University of Texas Southwestern Medical Center, Dallas, TX).[26] Mice expressing a Cre recombinase under control of *Albumin* promoter/enhancer elements (Alb-Cre; Jax#003574) and a second line of miR-21^{KO} mice (Jax#016856)[27] were from Jackson Laboratory. For HSC-specific ablation of Dicer, floxed Dicer1 mice (Dicer$^{f/f}$; Jax 006001)[28] were crossed with lecithin retinol acyltransferase (LRAT)-Cre mice.[1] When indicated, mice also expressed the Cre reporter ZsGreen (Jax#007906). Multidrug resistance

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protein 2 (MDR2)^{KO} mice[1] and mice carrying conditional alleles for phosphatase and tensin homolog (PTEN) and TGF beta receptor 2 (TGFBR2) have been described.[29] Further details are described in the Supporting Material.

HSC ISOLATION AND CULTURE

Mouse HSCs were isolated by *in situ* liver perfusion and cultured as described.[30-32] Further details are described in the Supporting Material.

MOUSE MODELS OF LIVER FIBROSIS

To induce toxic liver fibrosis, 8-week-old mice were administered CCl_4, (0.5 μL/gram body weight, dissolved in corn oil at a ratio 1:3) every 3 days for a total of eight doses given by intraperitoneal injection or gavage as indicated. Mice were euthanized 48 hours after the last dose. For cholestatic liver fibrosis, 8-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 a diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for 3 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, intraperitoneally) administered to male mice at day 15 postpartum, followed by 10 injections with CCl_4 (0.5 μL/g, intraperitoneally, once weekly) starting at week 6. In a second model, 6-week-old male C3H/HOJ mice were treated with DEN (100 μg/g, intraperitoneally), followed by 22 injections of CCl_4 (0.5 μL/g, intraperitoneally). Mice with Alb-Cre-mediated deletion of *Pten* and *Tgfbr2* were used as the 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 transposase, pT3-myrAKT, and pT3-YapS127A, as described.[33]

Additional methods are described in the Supporting Material.

Results

miR-21 INCREASES IN ACTIVATED HSCs AND IN THE FIBROTIC LIVER

Following liver injury, HSCs 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 HSCs from mice with toxic fibrosis induced by CCl_4 treatment or biliary liver injury induced by bile duct ligation (BDL) to quiescent HSCs. In addition to these two *in vivo* models of HSC activation, we included HSCs that were culture activated in plastic dishes (Supporting Fig. S1A,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; Supporting Table S1). 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-fold to 24-fold as determined by microarray and confirmatory quantitative polymerase chain reaction (qPCR; Fig. 1B,C). Moreover, miR-21 was one of four microRNAs that was significantly up-regulated (Fig. 1B), thus making it amenable to antimir-based therapeutic approaches. To confirm these findings, we also isolated HSCs 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 up-regulation 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; Supporting Fig. S1C). miR-21b was not detectable, while miR-21c was not changed in any of the employed HSC activation models (Supporting Fig. S1D). Taken together, these results indicate that the induction in miR-21 expression is a characteristic

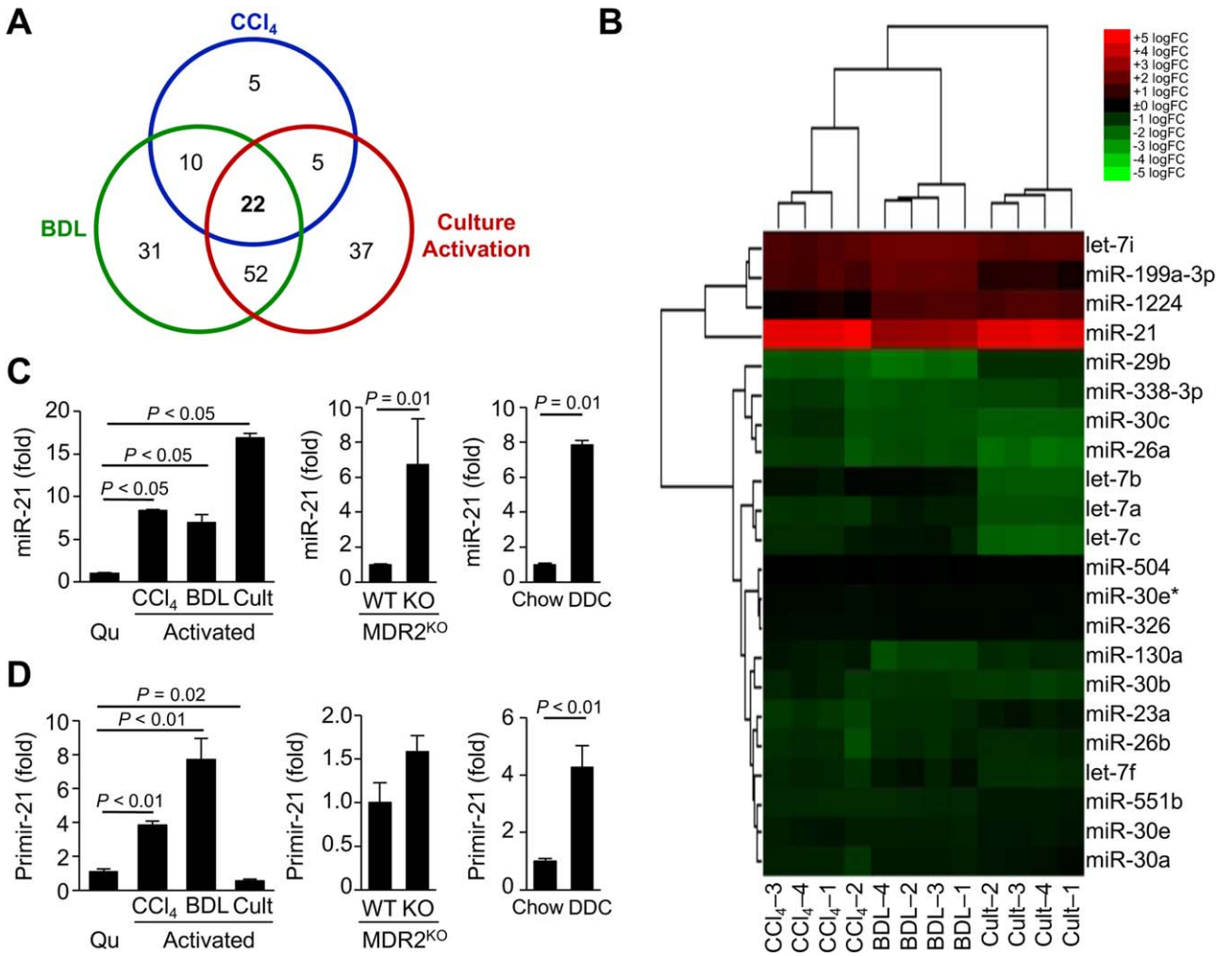

FIG. 1. HSC activation leads to changes in microRNA expression. (A) Venn diagram of the number of microRNAs that significantly change during CCl_4-, BDL-, and culture-induced HSC activation. [Correction added on February 12, 2018, after first online publication: The words "number of" were added to the prior sentence.] (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 HSCs and are expressed as fold induction compared to quiescent HSCs. HSCs were isolated from male mice; n = 4 for A,B; n = 4-6 for C,D. Data represent means ± SEM. Abbreviations: Cult, culture activated; Qu, quiescent HSC.

change during HSC activation and that miR-21 may represent an antifibrotic target.

miR-21 IS NOT REQUIRED FOR THE DEVELOPMENT OF TOXIC AND BILIARY LIVER FIBROSIS

We next used well-established models of toxic and biliary liver fibrosis to test the role of miR-21 in liver fibrogenesis. Following treatment of miR-$21^{KO(15)}$ mice and wild-type (WT) controls with eight injections of CCl_4, we did not observe significant differences in sirius red staining (Fig. 2A) or hepatic hydroxyproline content (Fig. 2B). Moreover, markers of HSC activation, including α-smooth muscle actin (α-SMA) protein and mRNA (encoded by *Acta2*), collagen type I alpha 1 (*Col1a1*), lysyl oxidase (*Lox*), and tissue inhibitor of metalloproteinase 1 (*Timp1*) mRNA, increased in CCl_4-treated livers but were similar between miR-21^{KO} mice and WT controls (Fig. 2C,D). mRNA and protein expression of the HSC marker desmin (*Des*) increased in livers from CCl_4-treated mice, indicating that HSCs expanded to a similar degree in miR-21^{KO} and WT mice during

fibrogenesis (Fig. 2D; Supporting Fig. S6D). There were also no differences in liver injury, as determined by plasma alanine aminotransferase (ALT) levels, or inflammation as assessed by tumor necrosis factor (*Tnf*) and chemokine (C-C motif) ligand 2 (*Ccl2*) mRNA expression (Fig. 2E,F). In addition to miR-21^{KO} mice, we employed inhibitory antisense DNA oligonucleotides (miR-21 knock-down, 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 the 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 nonfibrotic and fibrotic livers, respectively, compared to mice treated with scrambled control oligonucleotides (Supporting Fig. S2A,B). In addition, the expression of miR-21 target gene *Timp3*[34] was derepressed in nonfibrotic control livers but not in fibrotic livers, in which other regulatory mechanisms may predominate (Supporting Fig. S2B). To determine whether our antimir strategy decreased miR-21 in HSCs, we isolated HSCs from mice treated with miR-21KD antimir or scrambled control oligonucleotides. qPCR demonstrated an 80% decrease of miR-21 levels in HSCs from miR-21KD-treated mice (Supporting Fig. S2C), 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 (Supporting Fig. S2D,E) or the mRNA expression of HSC activation markers *Acta2*, *Col1a1*, and *Timp1* in whole liver (Supporting Fig. S2F). Moreover, miR-21KD antimirs did not reduce proliferation as determined by qPCR for marker of proliferation Ki-67 (*Mki67*) mRNA (Supporting Fig. S2F). 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 more brief 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 a 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 WT 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 laboratory. In addition, liver injury was not different between miR-21^{KO} and WT mice, as measured by plasma ALT levels (Fig 3B). As in our findings for 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 (Supporting Fig. S3A-C) or in female (Supporting Fig. S3D-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 compared to 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 (Supporting Fig. S4A-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 (Supporting Fig. S5A-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 (Supporting Fig. S6). In summary, our data from three

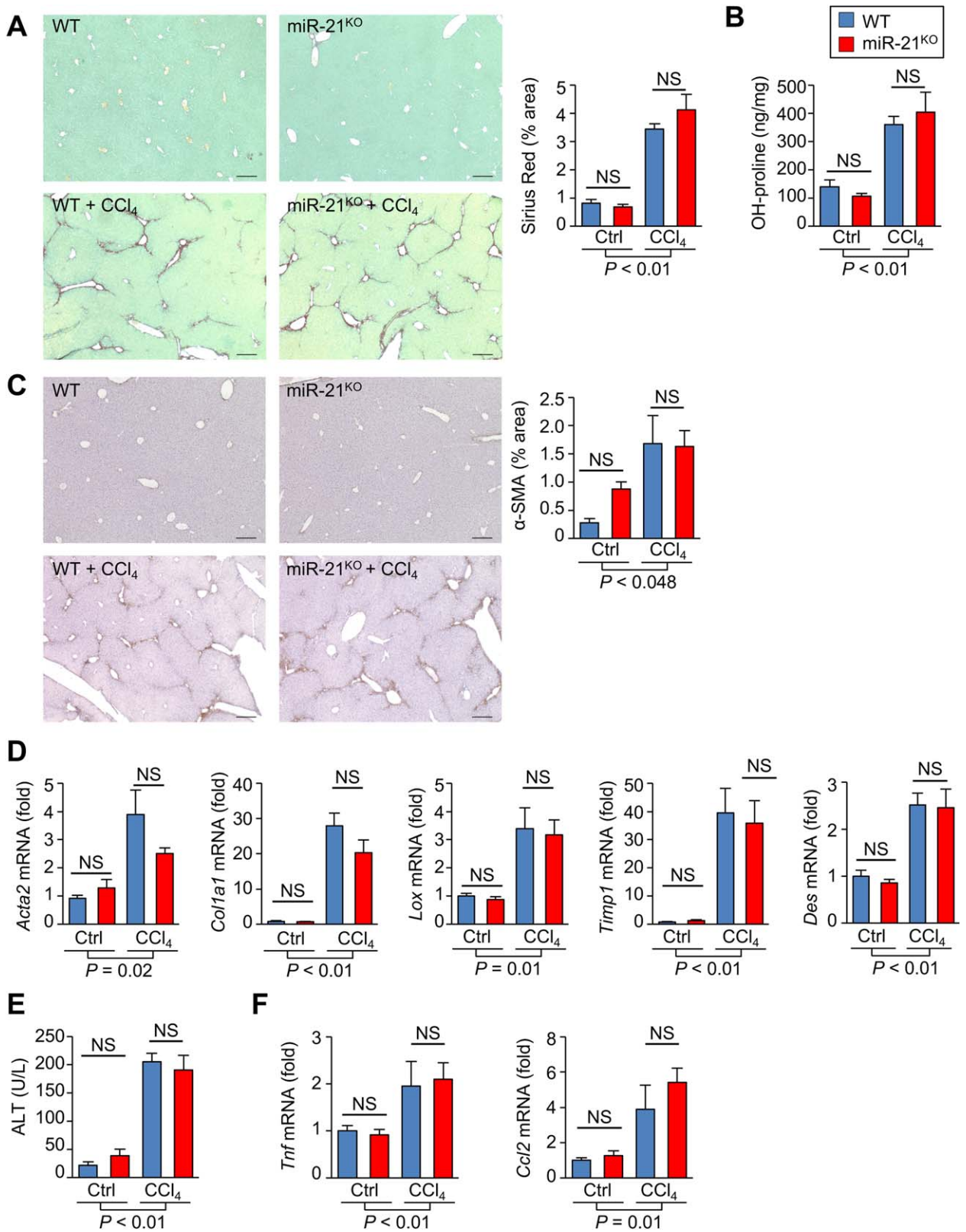

FIG. 2. miR-21 deletion does not prevent hepatotoxic fibrosis. WT and miR-21^{KO} female mice (n = 9/group) were treated with eight doses of CCl_4; untreated mice were used as control (n = 3/group). WT and miR-21^{KO} mice developed similar liver fibrosis as determined by (A) morphometric quantification of the sirius red-positive area or by (B) hepatic hydroxyproline content. miR-21 deletion did not affect HSC activation as assessed by (C) α-SMA immunohistochemistry or (D) qPCR for *Acta2*, *Col1a1*, *Lox*, and *Timp1* mRNA or HSC number evaluated by *Des* mRNA expression measured in liver samples. (E) Hepatocellular injury assessed by plasma ALT activity was similar in WT and KO mice. (F) 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. Data represent means ± SEM. Scale bar 200 μm. Abbreviations: Ctrl, control; NS, not statistically significant.

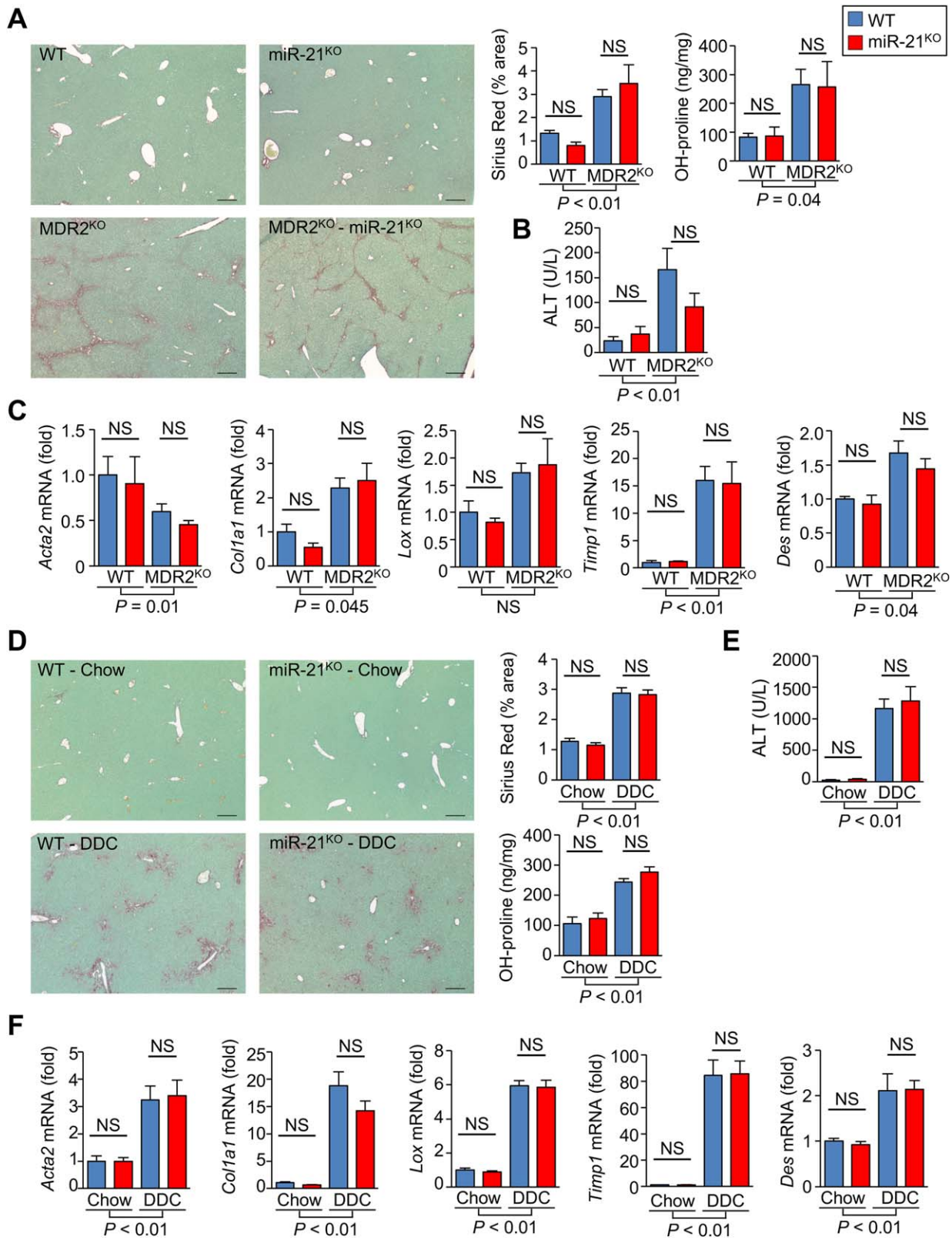

FIG. 3. Deletion of miR-21 does not prevent cholestatic liver fibrosis. Genetic deletion of miR-21 in MDR2^{KO} male mice (n = 9) did not reduce fibrosis as determined by (A) sirius red staining, (A, right panel) hepatic hydroxyproline content, (B) liver injury, (C) *Acta2, Col1a1, Lox,* and *Timp1* mRNA expression in liver, or HSC expansion assessed by *Des* mRNA expression when compared to MDR2^{KO} male mice that were WT for miR-21 (n = 11); mice WT 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 (D) sirius red staining, (D, right panel) hepatic hydroxyproline content, (E) plasma ALT activity, or (F) qPCR for *Acta2*, *Col1a1, Lox*, *Timp1,* and *Des* in liver; mice fed chow were use as controls (n = 6/group). Data represent means ± SEM. Scale bar 200 μm. Abbreviation: NS, not statistically significant.

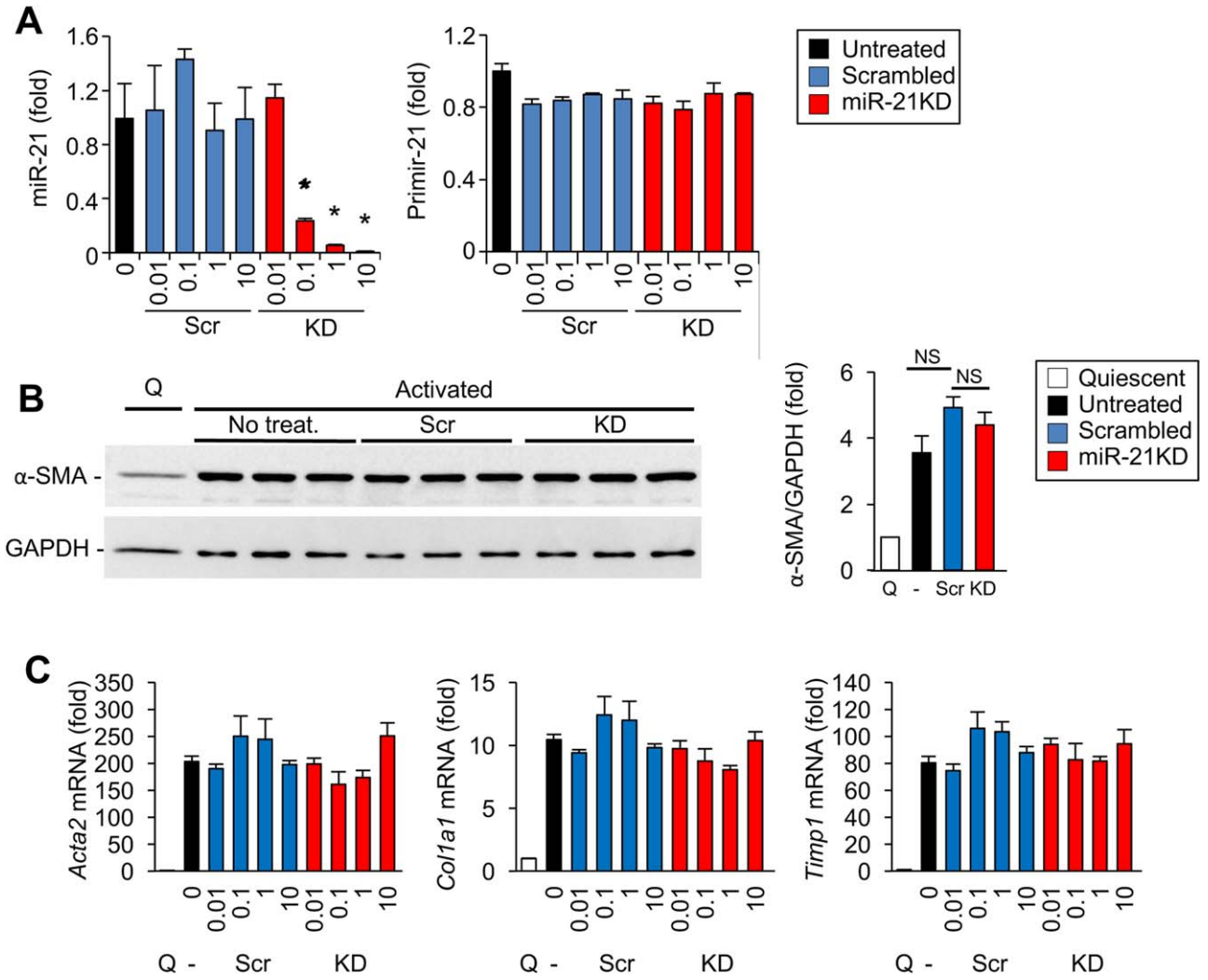

FIG. 4. Blocking miR-21 does not prevent mouse HSC activation in culture. Primary murine HSCs isolated from male Balb/C mice were plated, allowed to attach overnight, treated with miR-21KD or scrambled-sequence control oligonucleotides at indicated concentrations (in μM), and kept in culture for 5 days to induce activation. (A) miR-21KD treatment decreased miR-21 in a dose-dependent manner but not Primir-21. (B,C) Treatment with miR-21KD did not affect the activation of HSCs as evaluated by expression of (B) α-SMA determined by western blot or (C) HSC activation markers *Acta2*, *Col1a1*, and *Timp1* mRNA determined by qPCR; values are expressed as fold change compared to quiescent HSCs (n = 3). Data represent means ± SEM; $^*p < 0.05$ when compared with cells treated with the same concentration of scrambled-sequence oligonucleotides. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KD, miR-21KD; NS, not statistically significant; Q, quiescent HSC; Scr, scrambled-sequence.

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 HSCs

Next, we investigated whether miR-21 was necessary for HSC activation in cell culture as this model resulted in the strongest up-regulation of miR-21. Treatment of HSCs with miR-21KD antimirs decreased miR-21 in cultured HSCs in a dose-dependent manner (up to 99% at the highest dose) compared 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).

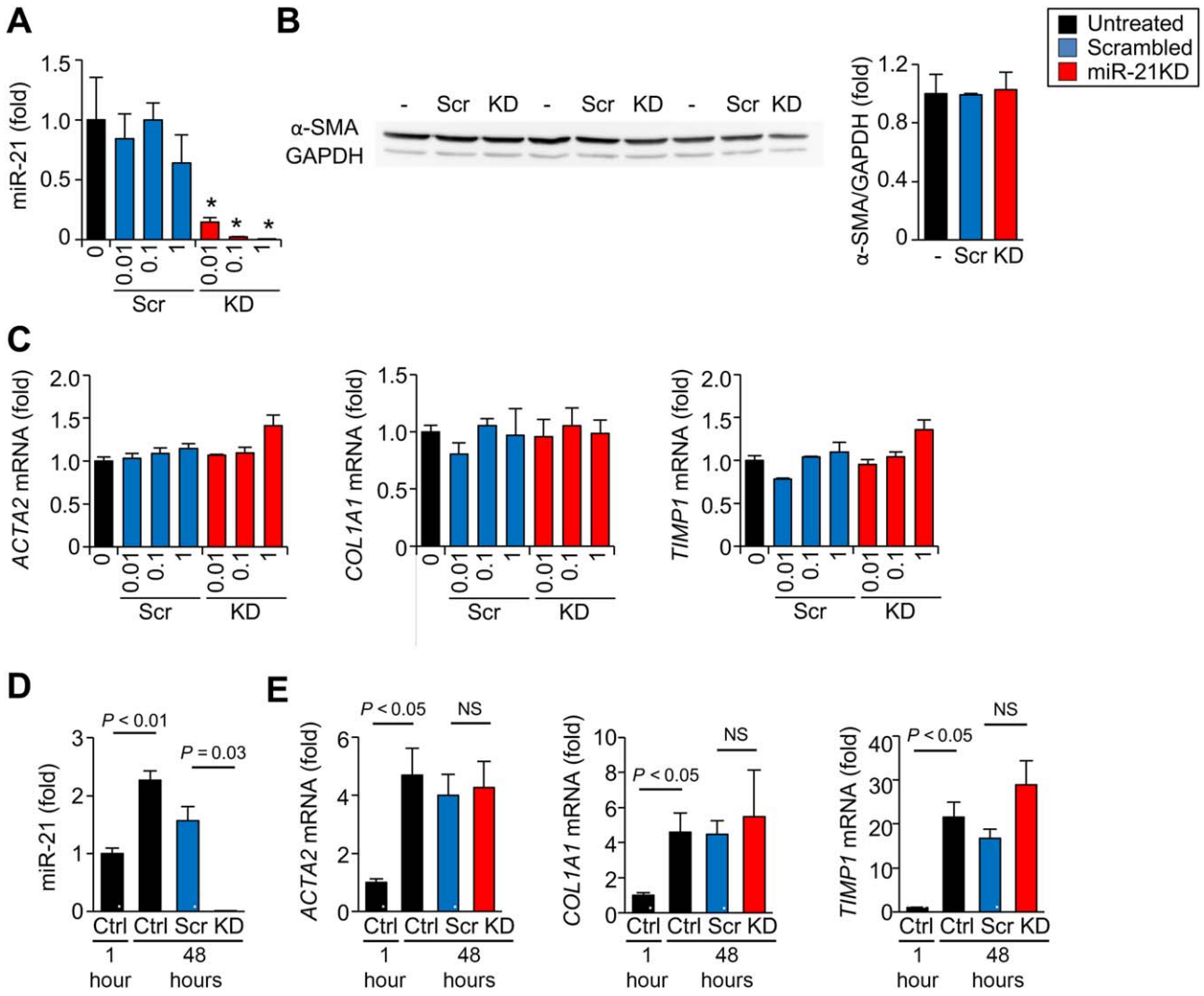

FIG. 5. Blocking miR-21 does not prevent human HSC activation. (A) Treatment of human primary HSCs with miR-21KD (0.01-1 μM) decreased miR-21 levels in a dose-dependent manner in comparison to control oligonucleotides (0.01-1 μM) but did not reduce (B) α-SMA protein expression (1μM) or (C) mRNA expression of *ACTA2*, *COL1A1,* or *TIMP1* (n = 3). (D) Treatment of human liver slices with miR-21KD (1 μM) decreased miR-21 but did not (E) reduce expression of *ACTA2*, *COL1A1*, or *TIMP1* mRNA (n = 7). Data represent means ± SEM; *p < 0.05 when compared with cells treated with the same concentration of scrambled-sequence oligonucleotides. Abbreviations: Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KD, miR-21KD; NS, not statistically significant; Scr, scrambled-sequence.

miR-21 IS NOT REQUIRED FOR THE ACTIVATION OF HUMAN HSCs IN CULTURE OR IN LIVER SLICES

To exclude the possibility that miR-21 exerts profibrotic effects in human HSCs that cannot be shown in murine models, we evaluated the effect of miR-21KD antimir in primary human HSCs. Treatment of human HSCs with miR-21KD antimir decreased miR-21 in a dose-dependent manner, achieving up to 99% reduction of miR-21 expression (Fig. 5A). However, similar to our data for murine HSCs, 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 i*n vivo*,[32,35] we additionally evaluated how miR-21 knockdown affects the activation of HSCs in liver slices, a model of fibrogenesis in which HSCs

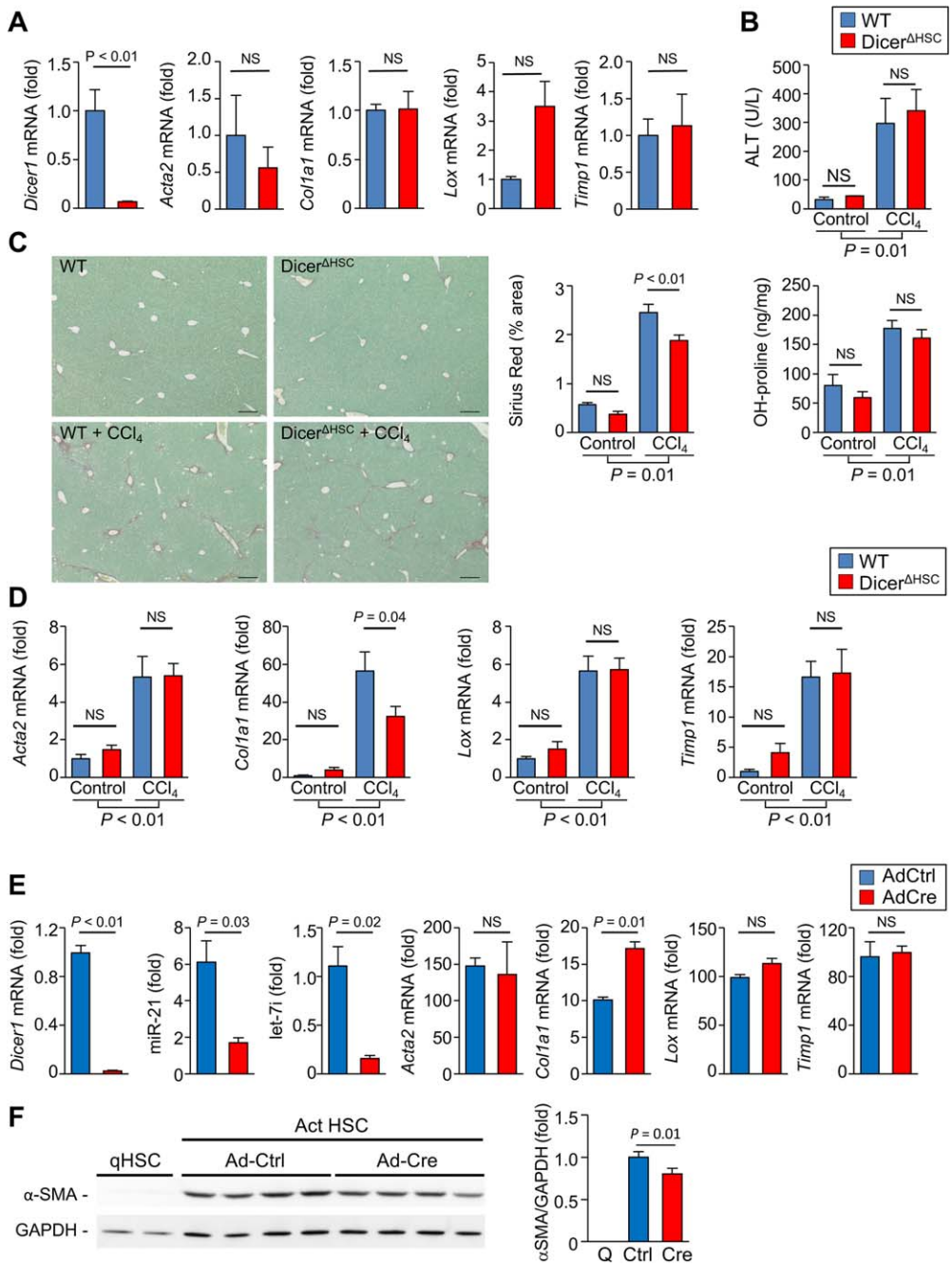

FIG. 6. Dicer1 deletion has minimal effects on HSC phenotype, HSC activation, and liver fibrosis. (A) HSCs were isolated from male WT or Dicer$^{\Delta HSC}$ mice, and the expression of Dicer1 and HSC activation genes was measured by qPCR in the isolated cells (n = 3). (B-D) Female WT (n = 9) and Dicer$^{\Delta HSC}$ (n = 10) mice were treated with four injections of CCl_4. (B) Liver injury was assessed by ALT activity; (C,D) liver fibrosis was assessed by (C) sirius red staining and hepatic hydroxyproline content and (D) mRNA expression of *Acta2*, *Col1a1*, *Lox,* and *Timp1* in whole livers. (E,F) HSCs isolated from Dicer floxed mice were transduced with control or Cre-expressing adenoviruses to delete Dicer and cultured for 7 days. (E) 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* and for (F) α-SMA protein expression (n = 4/group). Data represent means ± SEM. Values are expressed as fold change compared to quiescent HSCs. Scale bar 200 μm. Abbreviations: Δ, Dicer$^{\Delta HSC}$; Act HSC, activated HSC; Ad-Cre, Cre-expressing adenovirus; Ad-Ctrl, control adenovirus; Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NS, not statistically significant; Q, quiescent HSC; qHSC, quiescent 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 lethal-7 (let-7).[28] To further evaluate the importance of microRNAs in HSC activation and liver fibrosis, we generated mice with an 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 HSCs, 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; Supporting Fig. S7A). To determine the role of microRNA modulation by Dicer1 deletion, we first needed to exclude that Dicer1 deletion resulted in spontaneous activation of HSCs or that it severely disturbed the phenotype of HSCs. For this purpose, we isolated HSCs from $Dicer^{\Delta HSC}$ and $Dicer^{+/+}$ Lrat-Cre control mice. HSCs isolated from $Dicer^{\Delta HSC}$ mice had a normal phenotype with the presence of characteristic retinoid-containing lipid droplets (Supporting Fig. S7B, Day1, left panels). Moreover, RNA sequencing and qPCR showed no alterations in a panel of characteristic HSC genes, including heart and neural crest derivatives expressed 2 (*Hand2*), LIM homeobox 2 (*Lhx2*), and *Lrat,* or in fibrosis markers *Acta2, Col1a1,* and *Timp1*, with the exception of *Lox*, which was up-regulated in HSCs from $Dicer^{\Delta HSC}$ mice in our RNA sequencing data but did not show statistical significance in the qPCR (Fig. 6A; Supporting Fig. S7C; Supporting Table S2). Moreover, there was no change in the expression of *Pdgfrb* or *Tgfbr2* (Supporting Fig. S7C). When cultured to induce activation, HSCs isolated from $Dicer^{\Delta HSC}$ mice activated similarly to WT control cells, showing the characteristic fibroblastic phenotype with partial loss of retinoids (Supporting Fig. S7B, Day 5, right panels) and similar expression of α-SMA (Supporting Fig. S7D). To evaluate the effect of Dicer1 deletion in HSCs 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 no 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 (Supporting Fig. S7F), 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 HSCs *in vitro* and studied the effect on culture activation. For this purpose, we isolated HSCs from $Dicer^{f/f}$ mice, followed by transduction with an adenovirus encoding Cre recombinase (Ad5-cytomegalovirus[CMV]Cre) 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 compared to control adenovirus-infected HSCs (Fig. 6E). Moreover, *Dicer1* mRNA was already 98% reduced 72 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 HSCs from $Dicer^{\Delta HSC}$ mice that were culture activated for 5 days and displayed a significant decrease in *Dicer1* expression (Supporting Fig. S7D,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

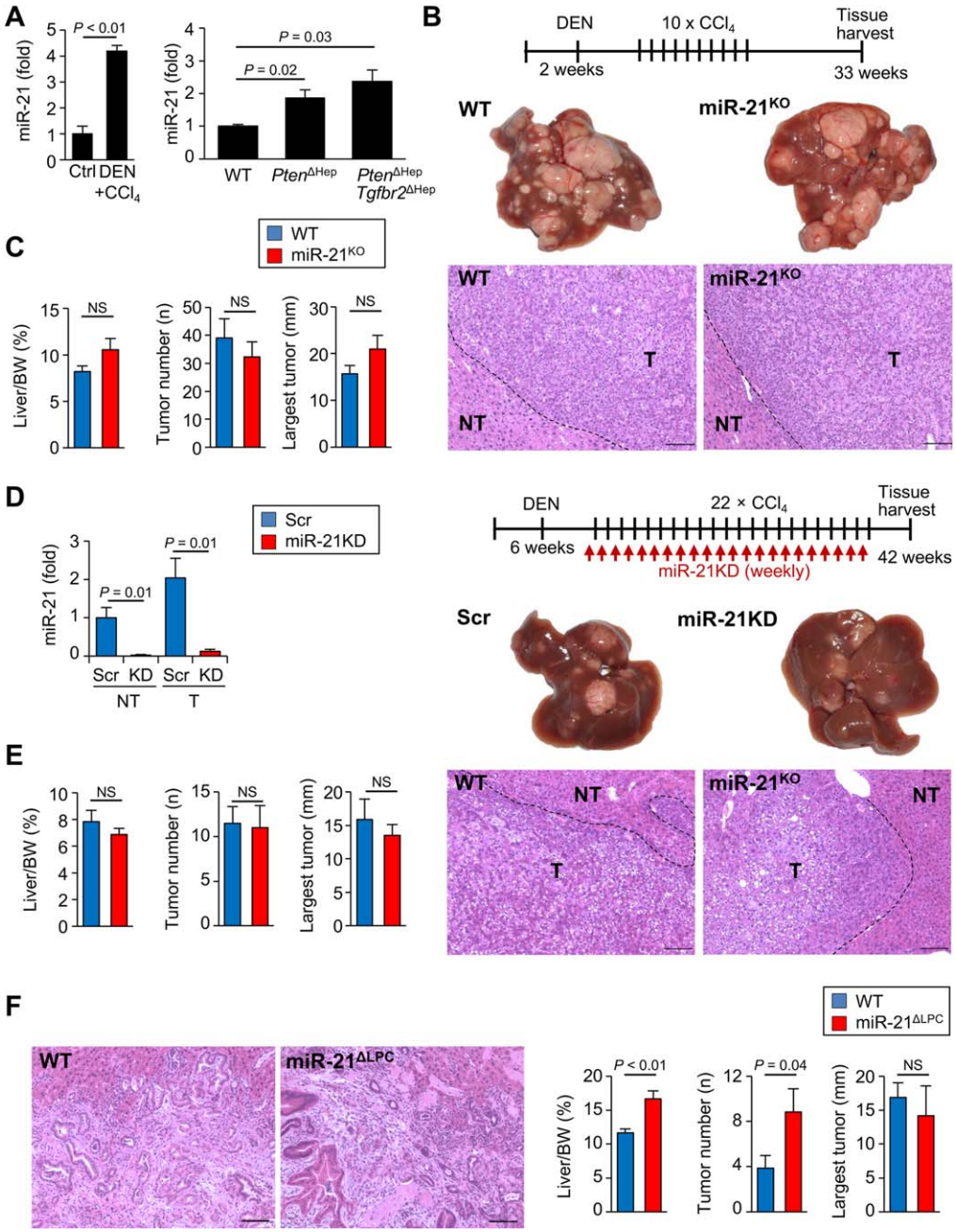

FIG. 7. miR-21 does not promote hepatic carcinogenesis. (A) miR-21 was determined by qPCR in liver tumors from DEN + CCl_4-treated mice (n = 3), from mice with liver-specific deletion of PTEN ($Pten^{\Delta LPC}$, n = 4), or PTEN and TGFBR2 (Pten $Tgfbr2^{\Delta LPC}$, n = 3) and expressed as fold induction compared to livers from nontreated WT mice (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; 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 (n = 8) or miR-21KD (n = 8). miR-21KD decreased miR-21 both in tumors and nontumor 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 compared 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. Data represent means ± SEM. Scale bar 100 μm. BW, body weight; Ctrl, control; KD, miR-21KD; NT, nontumor; Scr, scrambled oligonucleotides; T, tumor.

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] Up-regulation 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] As in 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. 7D).[44] Tumors showed increased expression of HCC marker glypican 3 (Gpc3) and proliferation marker Mki67 (Supporting Fig. S8A). Treatment with miR-21KD antimir efficiently decreased miR-21 in tumors and surrounding nontumor 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 (Supporting Fig. S8B).

Next, we evaluated whether miR-21 affects carcinogenesis in two cholangiocarcinoma models. First, we used a model of cholangiocarcinoma driven by deletion of PTEN and TGFBR2.[29] In this model, codeletion of miR-21 increased the tumor number and liver-to-body weight ratio but not tumor size (Fig. 7F). Second, we used a cholangiocarcinoma model driven by overexpression of activated protein kinase B (AKT) and yes-associated protein 1 (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, keratin 19 (Krt19)-positive area, and *Krt19* and prominin 1 (*Prom1*) mRNA expression (Supporting Fig. S9). These results are in agreement with the lack of any effect of miR-21KD on the hepatic expression of cancer-related target genes (Supporting Fig. S10).

To further determine the role of miR-21 in human liver cancer, we studied four liver cancer cell lines as well as two nontransformed controls *in vitro*. In HepG2 and PLC/PRF/5, transfection with miR-21KD decreased miR-21 levels 97% and 99%, respectively; however, it did not affect proliferation, apoptosis, or anchorage-independent growth (Supporting Fig. S11). Similarly, miR-21KD decreased miR-21 in HuCCT-1 and KMCH cells but did not affect those cancer properties (Supporting Fig. S12). In addition, transfection of hepatocyte cells transformed human liver epithelial-2 (THLE-2) and cholangiocyte cells H69 with miR-21 increased miR-21 levels but did not affect proliferation, apoptosis, and anchorage-independent growth (Supporting Fig. S13). 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 noncancer cells.

Discussion

Currently, there are no approved antifibrotic drugs for the treatment of liver fibrosis. Recent studies on lung, kidney, heart, and liver fibrosis have reported that blocking miR-21 exerts antifibrogenic 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 a 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 HSCs 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, BDL, or HSC culture activation. Moreover, we excluded that the lack of effect may have been species specific as evidenced by unaltered activation of primary human HSCs 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-21KD that may have been co-extracted with the RNA and interfered with the reverse transcription or qPCR assay (Supporting Fig. S14). Despite efficient knockdown of miR-21, we only observed a moderate increase in the expression of recognized miR-21 target *Timp3* mRNA (Supporting Fig. S2B)[14,26,34] and a trend toward increased sprouty1 (SPRY1) expression in some but not other experiments (data not shown). These variable and mostly absent effects are consistent with our finding of unaltered programmed cell death (PDCD)4 and SPRY1 protein in livers of miR-21^{KO} mice (data not shown) in our studies as well as previous studies in hearts of miR-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 up-regulated target gene expression in keratinocytes of the miR-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 a 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 up-regulation of miR-21b or miR-21c in either of the two miR-21^{KO} models (Supporting Fig. S14B,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 that 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 HSCs, and human liver slices all yielded similar data, supporting the conclusion that targeting miR-21 may not be a promising antifibrotic 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 HSCs *in vitro* and *in vivo*. By both approaches, we found a significant down-regulation of microRNA expression in HSCs 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 HSCs 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 HSCs suggests that microRNAs modulate the expression of specific genes in HSCs but that those genes are not involved in the programs that control the identity and activation of HSCs. 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 nonspecific 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 up-regulation of miR-21 in a wide range of tumors, including HCC and cholangiocarcinoma,[41-43] and a

potential role as an 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 and cell culture models. Despite an up-regulation of miR-21, we did not observe an effect of either miR-21 knockout or antimir treatment on the development of HCC or cholangiocarcinoma. In contrast with our results, Zhang et al.[23] reported that miR-21 promotes PTEN deletion-induced HCC. 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 downstream target Akt, which might not allow revealing 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 antitumorigenic 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 the focus should be on other microRNA as therapeutic targets.

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