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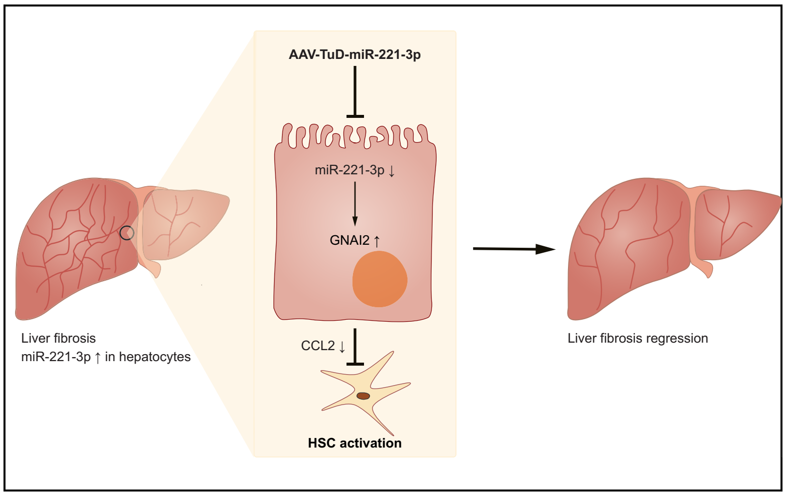
Hepatocyte-specific suppression of microRNA-221-3p mitigates liver fibrosis

Graphical abstract



Highlights

- Identification of microRNA-221-3p as a regulator of liver fibrosis *in vitro*.
- Inhibition of microRNA-221-3p in hepatocytes is capable of reducing liver fibrosis in mouse models.
- Treatment of primary human hepatocytes with microRNA-221 inhibitor suppresses activation of human myofibroblasts.

Authors

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Lay summary

Liver fibrosis majorly contributes to mortality resulting from various liver diseases. We discovered a small RNA known as miRNA-221-3p, whose downregulation in hepatocytes results in reduced liver fibrosis. Thus, inhibition of miRNA-221-3p may serve as one of the therapeutic approaches for treatment of liver fibrosis.

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Hepatocyte-specific suppression of microRNA-221-3p mitigates liver fibrosis

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Background & Aims: Fibrosis, a cardinal feature of a dysfunctional liver, significantly contributes to the ever-increasing mortality due to end-stage chronic liver diseases. The crosstalk between hepatocytes and hepatic stellate cells (HSCs) plays a key role in the progression of fibrosis. Although ample efforts have been devoted to elucidate the functions of HSCs during liver fibrosis, the regulatory functions of hepatocytes remain elusive.

Methods: Using an unbiased functional microRNA (miRNA) screening, we investigated the ability of hepatocytes to regulate fibrosis by fine-tuning gene expression via miRNA modulation. The *in vivo* functional analyses were performed by inhibiting miRNA in hepatocytes using adeno-associated virus in carbon-tetrachloride- and 3,5-di-diethoxycarbonyl-1,4-dihydrocolli dine-induced liver fibrosis.

Results: Blocking miRNA-221-3p function in hepatocytes during chronic liver injury facilitated recovery of the liver and faster resolution of the deposited extracellular matrix. Furthermore, we demonstrate that reduced secretion of C–C motif chemokine ligand 2, as a result of post-transcriptional regulation of GNAI2 (G protein alpha inhibiting activity polypeptide 2) by miRNA-221-3p, mitigates liver fibrosis.

Conclusions: Collectively, miRNA modulation in hepatocytes, an easy-to-target cell type in the liver, may serve as a potential therapeutic approach for liver fibrosis.

Lay summary: Liver fibrosis majorly contributes to mortality resulting from various liver diseases. We discovered a small RNA known as miRNA-221-3p, whose downregulation in hepatocytes results in reduced liver fibrosis. Thus, inhibition of miRNA-221-3p may serve as one of the therapeutic approaches for treatment of liver fibrosis.

Introduction

Liver fibrosis and cirrhosis contribute to more than 1 million deaths per year worldwide[1,2] (particularly, 170,000 per year in Europe[3] and 33,539 per year in the United States[4]). The underlying pathologies leading to fibrosis and subsequently cirrhosis are chronic virus infection, alcoholic steatohepatitis, and non-alcoholic steatohepatitis.[5] The manifestation of fibrosis is accompanied by the activation of quiescent HSCs, accumulation of excessive extracellular matrix, and hepatocyte dysfunction leading to liver failure.[6] While the majority of efforts have been concentrated on the elucidation of HSCs function during fibrosis, the regulatory functions of hepatocyte, the main parenchymal cells of the liver, remain to be understood further. Specifically, how gene expression alterations, particularly at the post-transcriptional level in hepatocytes, regulate fibrosis remains to be investigated. MiRNAs, one of the post-transcriptional regulators of gene expression,[7,8] have been reported to be deregulated in liver fibrosis.[7,9] The majority of studies have examined the functions of miRNAs in HSCs; however, identification and functional analysis of hepatocyte miRNAs, which are capable of regulating fibrosis, remain to be investigated. Elucidation of such key fibrosis-regulating miRNAs in hepatocytes, an easy-to-target cell type in the liver, would allow development of successful therapeutics for liver fibrosis.

Keywords: miRNA; Fibrosis; Hepatic stellate cells; miRNA-221-3p; Chemokine ligands.

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Materials and methods

Animals

We used 8–12-week-old male BALB/c mice (Charles River, Sulzfeld, Germany) for all *in vivo* studies. Mice (5 in 1 cage) were provided free access to food and water and kept in a colony room on a 12:12-hour light and dark cycle. All experiments, within this study, were performed according to animal protocol (TVA 17/2658), which was approved by Hannover Medical School and authorities of Lower Saxony State, Germany.

miRNA mimic library screening

The 96-well inserts (Corning 3392, Darmstadt, Germany) were coated with 50 µg/ml collagen (Roche, Mannheim, Germany) at room temperature for 1 hour. After discarding the collagen solution, 6,500 primary mouse hepatocytes were seeded onto each air-dried insert and were cultured with hepatocyte culture medium (HCM) overnight. A total of 302 miRNAs, which are conserved between human and mouse, were applied for the transfection in a final concentration of 25 nM miRNA per well. At 16 hours post transfection, the transfected primary mouse hepatocytes were placed on top of a monolayer of mHSC-Col1a1-GFP cells (7,000 cells per well) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum, 4 mM L-glutamine, and 1% penicillin–streptomycin for further incubation at 37 °C. The green fluorescent protein (GFP) intensity of mHSC-Col1a1-GFP cells was measured after 48 hours of co-culture.

Mouse model of liver fibrosis

Animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of the Hannover Medical School, Germany. BALB/c mice (8–12-week-old) were purchased from the central animal facility of the Hannover Medical School, Germany. To establish liver fibrosis *in vivo*, BALB/c mice were injected with 4 µl of 10% CCl_4–olive oil (Merck (Sigma), Darmstadt, Germany) per gram of mouse body weight, 2 times a week for 8 weeks. Mice in the control group were injected with only olive oil.

In situ hybridisation analysis

In situ hybridisation analysis was performed on 4-µm paraffin sections according to a standard procedure with digoxigenin-labelled antisense riboprobes[10–12] with following exceptions: hybridisation occurred at 54 °C and washing in 50% formamide/2 × saline-sodium citrate at 50 °C. The final concentration of the *miR-221-3p*-probe (Exiqon, Vedbaek, Denmark) was 40 nM. As a control we used 0.2 nM *U6*-probe.

AAV8 virus production

293T cells, with 60% confluency, were transfected with 25 µg pDP8.ape (Plasmid Factory, Bielefeld, Germany) and 25 µg pAAV-Ttr-control using the calcium phosphate method. To construct pAAV-Ttr-TuD, we synthesised TuD-miR-221-3p[13] with restriction sites *Sac*II and *Not*I and ligated this into a pAAV-Ttr plasmid. Adeno-associated virus serotype 8 (AAV8) virus was produced as previously described.[14,15] Viral titre was determined by quantitative polymerase chain reaction (qPCR) using primers targeting the *Ttr* promoter.

Primary HSC isolation

The procedure was performed as previously described.[16] In brief, 5 to 10 livers from mice with a minimum age of 35 weeks were perfused with Pronase E solution and Collagenase P solution at a flow speed of 6.5 ml/minute for 4.5 minutes at 37 °C. All perfused mouse livers were collected and digested *in vitro* with 50 ml Pronase E–Collagenase P solution and 1 ml DNase I at 37 °C for 20 minutes. The cell suspension was filtered through 100-µm cell strainers and centrifuged at 600*g* at 4 °C for 10 minutes. After discarding the supernatant, the cells were resuspended in Gey's Balanced Salt Solution-B (GBSS-B) and 150 µl DNase I solution was added to the cell suspension. The cell suspension was centrifuged at 600*g* at 4 °C for 10 minutes. After carefully discarding the supernatant, the cells were suspended with 36 ml GBSS-B buffer and 150 µl DNase I solution was added to the solution. The 14 ml Nycodenz solution (8 g Nycodenz with 28 ml GBSS-A buffer) was added to the cell suspension to reach an 8% Nycodenz gradient. The gradients were centrifuged at 1,500*g* at 4 °C for 15 minutes without break. Primary HSCs were harvested from the interphase of the gradients. Primary human myofibroblasts were purchased from Innoprot (Bizkaia, Spain). Primary HSCs were cultured with DMEM supplemented with 10% heat-inactivated foetal bovine serum, 1% penicillin–streptomycin, and 4 mM L-glutamine.

Primary hepatocyte transfection

One million primary mouse hepatocytes were seeded per well of collagen-coated six-well plates. Four hours after seeding, the medium was changed to fresh HCM. Virofect-enhanced hepatocytes were transfected with 100 nM miRNA mimics or scramble control (Scr) with TargeFect F-2 (Targeting system, EI Cajon, CA, USA). The medium was changed to fresh HCM 8 hours post transfection. Cell pellets were collected 48 hours after transfection. Primary human hepatocytes were isolated from liver tissue obtained from patients undergoing partial hepatectomy and according to written informed consent approved by the ethics commission of Hannover Medical School (Ethics-commission of Hannover Medical School, No. 252-2008), as previously reported.[17] Primary human hepatocytes from fibrotic livers and control (non-fibrotic livers) were purchased from PRIMACYT GmbH.

Co-culture of primary mouse hepatocytes and primary mouse HSCs

A total of 100,000 primary hepatocytes were seeded per transwell insert (Merck (Corning 3494), Darmstadt, Germany) for overnight culture. Hepatocytes were then transfected with either 1 µg control vector (pCMV-GFP) or 1 µg GNAI2 expression vector (pCMV-GNAI2-GFP, Biocat, Heidelberg, Germany). After 12–16 hours of transfection, the insert was placed on the top of primary mouse HSCs (200,000 cells per well) for further 48 hours co-culture. The primary mouse HSCs were fixed with 4% paraformaldehyde or the cell pellets were collected for further experiments.

Fluorescence-activated cell sorting analyses for CD11b and F4/80

Mononuclear cell isolation from the liver was performed as previously described.[16,18] After *in situ* perfusion and *in vitro* digestion of the liver, cells were filtered through 70-µm cell strainers and centrifuged at 300*g* for 8 minutes at 4 °C. The cell pellet was resuspended in 33% Percoll and centrifuged at 500*g* for 15 minutes at room temperature. Following red blood cell lysis (Merck, (Sigma) Darmstadt, Germany), cells were immunolabelled with fluorochrome-conjugated antibodies (BioLegend and BD, Heidelberg, Germany) for flow-cytometry analysis (LSR II Sorb, Becton Dickinson, Heidelberg, Germany).

For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Statistical analysis

Significance was determined with the two-tailed Student's *t* test for comparison of 2 groups. Significance between multiple groups was determined by one-way ANOVA. All $p < 0.05$ values were considered significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Error bars represent ± standard error of the mean (SEM).

Results

Functional miRNA screening identifies miR-221-3p as a potential fibrosis regulator

To identify miRNAs whose modulation in primary hepatocytes is capable of regulating liver fibrosis, we undertook an miRNA screening approach, wherein we examined a fibrogenic role of 302 miRNAs. These miRNAs represent a library of conserved miRNAs between mouse and human. Our miRNA screening required use of a HSC line, which allows measurement of fibrosis-related markers in a high-throughput manner. For this purpose, we used mouse HSCs, which express GFP under the transcriptional control of collagen 1 promoter/enhancer (henceforth referred to as Col-GFP HSCs).[19] Thus, we first transfected primary mouse hepatocytes with individual miRNAs and subsequently co-cultured them with Col-GFP HSCs (Fig. 1A). After 48 hours, we measured GFP intensity as surrogate readout of fibrogenesis (Fig. 1B). The GFP intensity of co-cultured cells transfected with Scr was set as 1. Based on this screening, we selected 15 miRNAs, which demonstrated maximum elevation in GFP intensity. We then validated those 15 miRNAs in 3 independent co-culture experiments; 12 of 15 miRNAs showed similar results compared with those obtained from our miRNA screen. Notably, 6 (miR-151a-5p, -374b-5p, -200b-3p, -219-2-3p, -221-3p, and -30c-1-3p) of these 12 miRNAs significantly ($p < 0.05$) increased GFP intensity (Fig. 1C), indicating that these 6 miRNAs may promote fibrogenesis. In addition, we found that 2 miRNAs (miR-17-5p and miR-7-1-3p), whose transfection in primary hepatocytes reduced GFP intensity (Fig. 1C), may serve as negative regulator of fibrosis.

Tissue inhibitor of metalloproteinases 1 (TIMP1) is secreted by activated HSCs and inhibits metalloproteinases to suppress extracellular matrix degradation.[20,21] We therefore measured levels of secreted TIMP1, as an unbiased second parameter to identify miRNAs, which are capable of regulating fibrosis. Based on 3 independent experiments, we found that transfection of miR-221-3p and miR-17-5p mimics led to significant increase in TIMP1 levels as shown by enzyme-linked immunosorbent assay (ELISA; Fig. 1D and Fig. S1A). We then confirmed elevation in TIMP1 levels in primary mouse HSCs (instead of Col-GFP HSCs) that were co-cultured with primary hepatocytes transfected with either miR-221-3p (Fig. 1E) or miR-17-5p mimic (Fig. S1B). The transfection of miR-221-3p (Fig. 1E) but not miR-17-5p mimics (Fig. S1B) led to significant elevation in TIMP1 levels, indicating that only gain of miR-221-3p in hepatocytes affected fibrogenesis from co-cultured primary mouse HSCs. Furthermore, miR-221-3p mimic transfection led to higher mRNA levels of characteristic fibrogenic markers such as *Col1a1*, *Acta2,* and *Tgfbr1* (Fig. 1E). To rule out whether miRNA-221-3p is transferred from primary mouse hepatocytes, which were transfected with miR-221-3p mimic, to primary HSCs, we analysed miR-221-3p expression in both cell types. We observed significantly higher levels of miR-221-3p only in primary hepatocytes but not in primary HSCs (Fig. S1C), thus excluding any passive transfer of miRNA from hepatocytes to HSCs in our co-culture setting. Therefore, based on our functional miRNA library screening, and subsequent analyses of TIMP1 secretion and fibrogenic markers, we identified miR-221-3p as a potential fibrosis regulator and selected it for further study.

miR-221-3p is upregulated in primary hepatocytes during liver fibrosis

Our screen indicated a potential fibrogenic role of miR-221-3p mimic in cultured primary hepatocytes *in vitro*. To understand the relevance of this finding in liver fibrosis, we determined miR-221-3p levels in a mouse model of liver fibrosis, which was induced by injecting BALB/c mice with CCl_4 for 8 weeks. At first, we confirmed liver fibrosis by Sirius Red staining (Fig. S2A) and mRNA expression analyses of profibrogenic markers such as *Col1a1*, *Acta2,* and *Tgfbr1*, which were found to be increased in the fibrotic mouse livers (Fig. S2B).

Because our screen suggested a pro-fibrogenic role of miR-221-3p, we reasoned that miR-221-3p levels should be deregulated during liver fibrosis. We therefore measured miR-221-3p levels in livers of mice injected with CCl_4. We observed a high miR-221-3p expression in fibrotic liver tissues by *in situ* hybridisation (Fig. 2A). The increased levels of miR-221-3p in fibrotic liver tissues were confirmed by qPCR analysis (Fig. 2B). To further confirm whether miR-221-3p expression is enhanced in hepatocytes, we purified primary hepatocytes from fibrotic livers by liver perfusion followed by density gradient centrifugation. We observed significant upregulation of miR-221-3p in hepatocytes (10.69-fold) isolated from the fibrotic mouse liver tissues compared with hepatocytes obtained from normal healthy liver (Fig. 2C). In addition to hepatocytes, we found significant upregulation of miR-221-3p in HSCs as reported previously,[22] and a mild though not significant increase in Kupffer cells isolated from fibrotic livers.

Because miR-221-3p is upregulated in hepatocytes during liver fibrosis, we investigated the effect of miR-221-3p inhibition in hepatocytes on liver fibrosis. To effectively knock down the expression of miR-221-3p *in vivo* and *in vitro*, we used Tough Decoys (TuDs). TuDs possess a hairpin structure containing 2 bulged miRNA targeting sites, which contribute to imperfect base pairing with the miRNA of interest.[13,23] TuDs have previously been reported to inhibit miRNA function efficiently.[13] We first confirmed downregulation of miR-221-3p upon transfection of primary hepatocytes with TuD-miR-221-3p (Fig. 2D). Importantly, to examine efficacy of TuD-miR-221-3p at the functional level, we analysed the protein level of PUMA (encoded by the *BBC3* gene), a known target of miR-221-3p,[15] in primary hepatocytes transfected with TuD-miR-221-3p. We observed increased PUMA levels upon TuD-miR-221-3p transfection (Fig. 2E). These results suggest that the TuD-miR-221-3p is able to inhibit miR-221-3p expression efficiently at the functional level as well.

MiR-221-3p inhibition in hepatocytes leads to faster resolution of liver fibrosis in vivo

Because our functional miRNA screening resulted in identification of miR-221-3p and its expression was also upregulated in

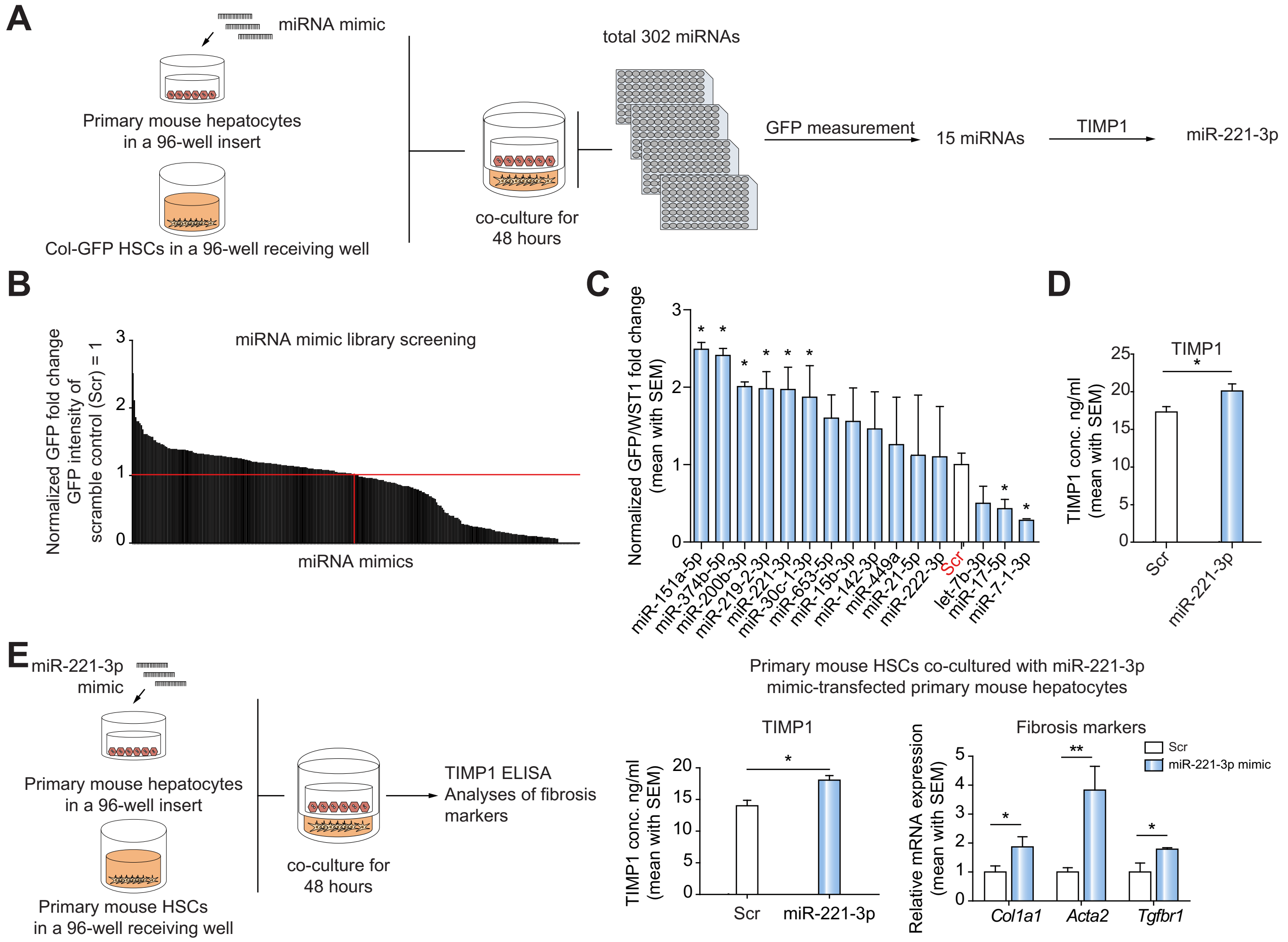


Fig. 1. The miRNA mimic library screening identifies miR-221-3p as potential fibrosis regulator. (A) Scheme showing the experimental design of miRNA mimic library screening and subsequent steps to identify miRNAs, whose modulation in primary hepatocytes is capable of regulating fibrosis. (B) The fold change in GFP intensity upon transfection of 302 miRNA mimics is depicted as readout of fibrosis. The GFP intensity of scramble control (Scr) is set as 1 (n = 3). (C) Fifteen miRNAs transfected in primary mouse hepatocytes contributed to significantly increased GFP/WST1 intensity of Col-GFP HSCs than respective Scr. The GFP/WST1 intensity of scramble is set as 1 (n = 4). (D) ELISA results show that miR-221-3p mimic transfection led to higher TIMP1 concentration than that of scramble. (E) Scheme showing the experimental design of confirmatory experiments performed for validation of miR-221-3p. Primary hepatocytes were transfected with miR-221-3p mimic and subsequently co-cultured with primary mouse HSCs. TIMP1 ELISA and qPCR analyses revealed significantly upregulated TIMP1 levels and fibrosis markers, respectively. Error bars represent ± SEM. One-way ANOVA was used for statistical evaluation. (*p <0.05; **p <0.01). ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; HSC, hepatic stellate cell; miRNA, microRNA; qPCR, quantitative PCR; TIMP1, Tissue inhibitor of metalloproteinases 1.

hepatocytes during fibrosis, we hypothesised that inhibition of miR-221-3p in hepatocytes of fibrotic livers would ameliorate liver fibrosis. To test this hypothesis, we aimed to inhibit miR-221-3p in hepatocytes of fibrotic livers by injecting mice with 1×10^{10} AAV8 encoding TuD-miR-221-3p (AAV TuD) at 6th and 8th week of CCl_4 treatment (Fig. 3A). Mice were killed 1 week after the second AAV injection. To suppress miR-221-3p specifically in hepatocytes, we expressed TuD-miR-221-3p under the transcriptional control of hepatocyte-specific *transthyretin* (*Ttr*) promoter, which significantly downregulated miR-221-3p in hepatocytes (Fig. 3B). To confirm functional repression of miR-221-3p *in vivo*, we detected protein levels of PUMA by immunoblotting. PUMA was found to be upregulated in hepatocytes isolated from fibrotic livers of mice injected with AAV TuD, indicating functional repression of miR-221-3p (Fig. 3C). After confirming miR-221-3p inhibition *in vivo*, we determined fibrosis by histology and Sirius Red staining. We observed reduced liver damage and less Sirius Red-stained areas in livers of mice injected with AAV TuD compared with respective controls (Fig. 3D). Correlating with the results of Sirius Red staining, reduced cellular infiltration was also observed in liver tissues of mice injected with AAV TuD compared with those injected with AAV control (Fig. 3D). Moreover, the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin were significantly downregulated in serum samples of mice injected with AAV TuD compared with the respective controls (Fig. 3E), suggesting reduced liver injury upon miR-221-3p suppression *in vivo*.

Because the development of fibrosis is regulated by HSCs, we investigated whether the markers of HSC activation decreased upon miR-221-3p inhibition. The mRNA levels of profibrogenic markers, *Col1a1*, *Acta2*, and *Tgfbr1* (Fig. 3F), and the total collagen content (Fig. 3G) were significantly downregulated in mice injected with AAV TuD. Quiescent HSCs are activated in

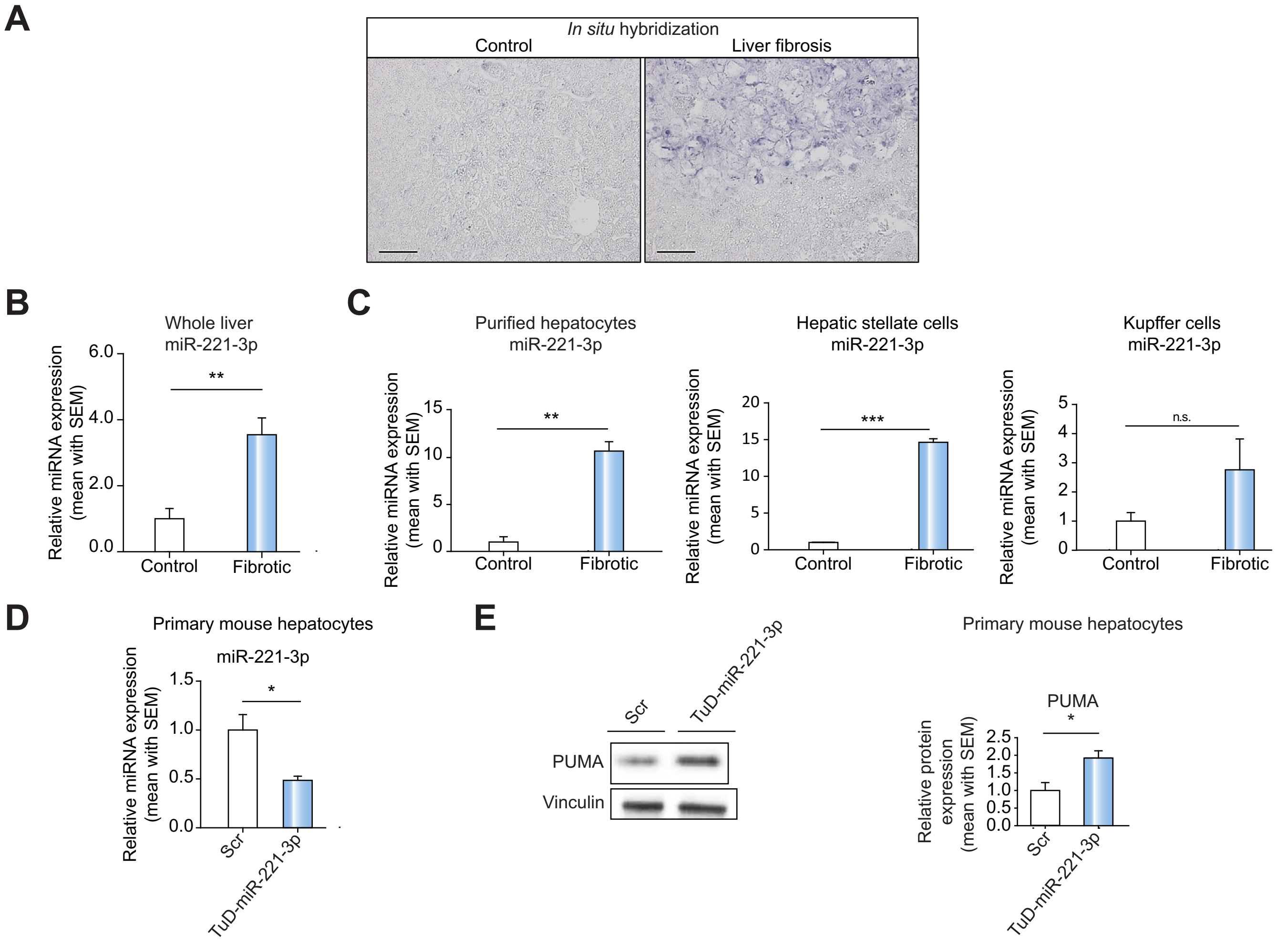


Fig. 2. MiR-221-3p is upregulated in liver fibrosis. (A) *In situ* hybridisation indicates higher miR-221-3p expression in fibrotic mouse liver tissues compared with miR-221-3p expression in control (healthy mouse livers). qPCR confirms significantly increased miR-221-3p expression in (B) liver and (C) hepatocytes and HSCs, but not in Kupffer cells of a CCl_4-induced mouse liver fibrosis model compared with non-fibrotic livers (n = 3 mice/group). (D) Significant downregulation of miR-221-3p by TuD-miR-221-3p in primary hepatocytes led to (E) upregulation of PUMA, a known miR-221-3p target gene *in vitro* (n = 3). Scale bars: 50 µm. Error bars represent ± SEM. Two-tailed Student's *t* test was used for statistical evaluation ($^*p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001). HSC, hepatic stellate cell; miRNA, microRNA; qPCR, quantitative PCR; Scr, scramble control. (This figure appears in colour on the web.)

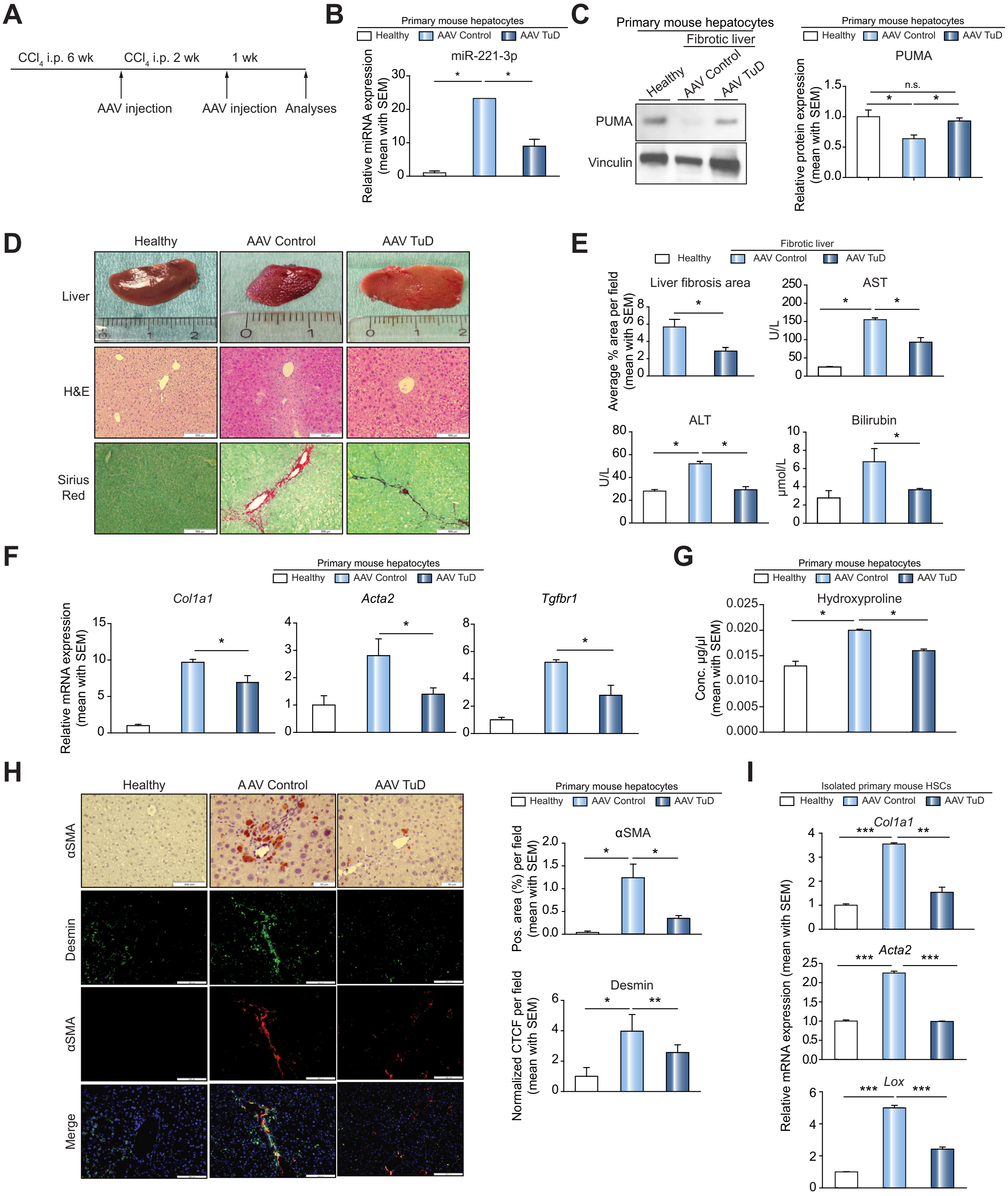
response to injury by differentiating into α-smooth muscle actin (α-SMA)-expressing myofibroblasts.[24] We observed significantly lower α-SMA- and desmin-positive cells (Fig. 3H) in the livers of mice injected with AAV TuD, suggesting reduced fibrosis upon miR-221-3p inhibition. To further confirm that miR-221 inhibition in hepatocytes reduces fibrosis, we isolated HSCs from mice injected with either AAV TuD or AAV control, and measured the expression of *Col1a1*, *Lox*, and *Acta2*, which are considered markers of HSC activation. We found a significantly lower expression of *Col1a1*, *Lox*, and *Acta2* (Fig. 3I), indicating reduced fibrosis upon inhibition of miR-221-3p in hepatocytes. Taken together, *in vivo* inhibition of miR-221-3p in fibrotic liver by AAV TuD ameliorates liver fibrosis.

To rule out adverse effects of miR-221-3p suppression in normal livers, we performed haematoxylin–eosin and Sirius Red staining and measured ALT, AST, and bilirubin levels in healthy mice injected with AAV control and AAV TuD. Haematoxylin–eosin, Sirius Red staining, mRNA expression of fibrogenic markers, AST, ALT, and bilirubin levels indicated absence of any liver damage upon administration of AAV TuD (Fig. S3). These results suggest that AAV TuD neither caused damage nor induced liver fibrosis in healthy livers, and hence, may be suitable for miR-221-3p inhibition in the clinic.

CYP2E1 is considered a key metabolic enzyme for CCl_4-induced toxicity. Specifically, CYP2E1 deficiency protects mice against CCl_4-induced liver injury.[25] Therefore, to rule out the possibility that AAV TuD administration reduced *Cyp2e1* levels and hence exhibited lower fibrosis, we measured *Cyp2e1* mRNA by qPCR. Both AAV TuD and AAV control groups of mice showed similar levels of *Cyp2e1* (Fig. S4), indicating that *Cyp2e1* levels did not contribute to attenuated fibrosis in AAV TuD-injected mice.

Post-transcriptional regulation of Gnai2 by miR-221-3p during liver fibrosis

To investigate the underlying mechanism leading to amelioration of liver fibrosis upon miR-221-3p inhibition, we performed *in silico* analyses to identify potential miR-221-3p targets that may play a role in liver fibrosis. We used algorithms such as TargetScan (http://www.targetscan.org/), miRDB (http://mirdb.org/miRDB/), and PicTar (http://pictar.mdc-berlin.de/) to narrow down the potential miR-221-3p target gene list (Fig. S5A). To


A
CCl4 i.p. 6 wk
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Desmin
Normalized CTCF per field (mean with SEM)
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Relative mRNA expression (mean with SEM)

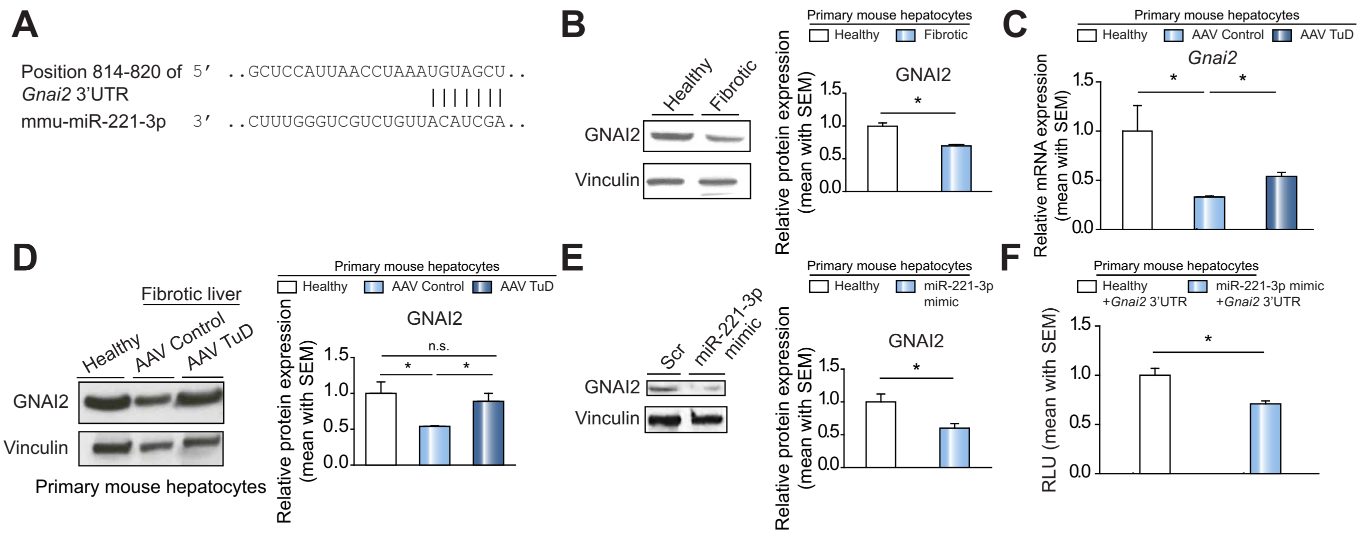

Fig. 4. *Gnai2* is a novel target gene of miR-221-3p. (A) A 3′UTR sequence of *Gnai2* targeted by miR-221-3p. (B) GNAI2 is significantly downregulated in primary hepatocytes isolated from fibrotic liver tissues *in vivo*. AAV TuD injection leads to upregulation of *Gnai2* (C) mRNA and (D) protein levels in isolated primary hepatocytes (n = 7 mice/group). (E) Transfection of miR-221-3p mimic in primary hepatocytes leads to a decrease in GNAI2 protein levels compared with respective scramble control (n = 5). (F) Dual luciferase reporter assay shows that miR-221-3p directly binds to 3′UTR of *Gnai2*, suggesting that *Gnai2* is a direct target of miR-221-3p. Data are shown as fold change and compared with the control group set as 1. Error bars represent ± SEM. One-way ANOVA was used for statistical evaluation. (*p <0.05). AAV, adeno-associated virus; RLU, relative luminescence units (n = 4); Scr, scramble control; TuD, Tough Decoy; UTR, untranslated region.

cluster the functions of the candidate genes, we applied DAVID (https://david.ncifcrf.gov/) analyses for functional gene annotation (Fig. S5B) to uncover mechanism of amelioration of liver fibrosis upon miR-221-3p. We identified *Gnai2* (G protein alpha inhibiting activity polypeptide 2), which has previously been suggested to play key roles in chronic liver diseases,[26] as a promising candidate. The 3′ untranslated region (3′UTR) of *Gnai2* contains a binding site for miR-221-3p that is conserved in mouse and human (Fig. 4A). To address whether GNAI2 is regulated during liver fibrosis, we analysed GNAI2 protein levels in hepatocytes isolated from mice with healthy and fibrotic livers. The protein levels of GNAI2 were found to be decreased in hepatocytes from fibrotic livers compared with those from healthy livers (Fig. 4B). This indicates that the decrease in GNAI2 protein levels could be due to upregulated levels of miR-221-3p during liver fibrosis. Consistent with these findings, the *Gnai2* mRNA expression and the GNAI2 protein levels were derepressed in hepatocytes of mice with fibrotic livers injected with AAV TuD (Fig. 4C–D).

To determine whether *Gnai2* is indeed regulated by miR-221-3p, we transfected primary mouse hepatocytes with miR-221-3p mimic or Scr and analysed the protein levels by immunoblotting. GNAI2 protein levels decreased in cells transfected with miR-221-3p mimic compared with cells transfected with Scrs (Fig. 4E). To determine whether *Gnai2* is a *bona fide* target of miR-221-3p, we cloned the 3′UTR of *Gnai2* into a dual luciferase pmirGLO vector and co-transfected this vector with miR-221-3p mimic or Scr into primary mouse hepatocytes. The dual luciferase reporter assay showed that the luciferase activity significantly decreased in cells co-transfected with miR-221-3p mimic, confirming that *Gnai2* is a novel direct target of miR-221-3p (Fig. 4F). In addition to GNAI2, we examined the expression of GNAI3, a member of the Gi group that is frequently downregulated in liver diseases.[27] The protein level of GNAI3 was not regulated in hepatocytes upon inhibition of miR-221-3p *in vivo* (Fig. S5C), thus indicating that only *Gnai2* but not *Gnai3* is regulated by miR-221-3p at the post-transcriptional level.

MiR-221-3p inhibition in hepatocytes suppresses HSC activation via GNAI2

To address whether modulation of *Gnai2* in hepatocytes regulates the activation of HSCs, we established transwell co-cultures of primary mouse hepatocytes and primary mouse HSCs. Hepatocytes were cultured in transwells overnight and transfected with a GNAI2 expression vector (pCMV-GNAI2-

Fig. 3. Inhibition of miR-221-3p in hepatocytes ameliorates liver fibrosis *in vivo*. (A) Schematic of liver fibrosis induction and time points of AAV injections. (B) qPCR result shows that miR-221-3p is significantly downregulated in hepatocytes upon AAV TuD transfection (n = 5 mice/group). (C) Downregulation of miR-221-3p through AAV TuD leads to increase in PUMA as shown by immunoblotting (n = 3). The proteins were prepared from isolated hepatocytes. Two right lanes were loaded with protein lysates obtained from fibrotic livers. (D) AAV TuD suppresses liver fibrosis *in vivo* as observed by fewer micronodules (gross morphology), less cellular infiltration (HE staining), and significantly reduced area of liver fibrosis (Sirius Red staining; n = 25 fields/group). (E) Significantly lower levels of AST, ALT, and bilirubin in serum from mice injected with AAV TuD indicate reduced liver injury (n = 4 mice/group). Magnification: 100×. Error bars represent ± SEM (*p <0.05). (F) qPCR shows that the expression of fibrotic markers *Col1a1, Acta2,* and *Tgfbr1* was significantly downregulated (n = 4 mice/group). (G) The amount of collagen decreased in mice injected with AAV TuD. (H) The protein levels of α-SMA and desmin are significantly downregulated in fibrotic livers from mice injected with AAV TuD. The co-staining of α-SMA and desmin further indicated reduced fibrosis in mice injected with AAV TuD. (I) qPCR analyses show significantly lower levels of *Col1a1*, *Lox,* and *Acta2* in primary mouse HSCs isolated from the livers of mice injected with AAV TuD. Magnification: 200× (α-SMA and desmin; n = 25 fields/group). Error bars represent ± SEM. One-way ANOVA was used for statistical evaluation (*p <0.05; **p <0.01; ***p <0.001). AAV, adeno-associated virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CTCF, corrected total cell fluorescence; HE, haematoxylin–eosin; HSC, hepatic stellate cell; miRNA, microRNA; qPCR, quantitative PCR; SMA, smooth muscle actin; TuD, Tough Decoy.

GFP). After 12 hours of transfection, the transwells were placed on top of primary mouse HSCs. The upregulated expression of *Gnai2* mRNA in primary mouse hepatocytes was confirmed at 24 hours post transfection (Fig. S6A). The p75 neurotrophin receptor (p75NTR) is expressed at low levels in quiescent HSCs but is rapidly increased after its activation.[28,29] We then examined *p75Ntr, Acta2,* and *Des* mRNA expression levels by qPCR and protein levels by immunofluorescence staining. Both mRNA and protein levels of *p75Ntr, Acta2,* and *Des* were significantly downregulated in primary mouse HSCs co-cultured with GNAI2-overexpressing primary mouse hepatocytes (Fig. 5A,B).

To further confirm whether GNAI2 contributes to the antifibrotic effect observed upon miR-221-3p inhibition, we co-cultured primary mouse HSCs with primary mouse hepatocytes,

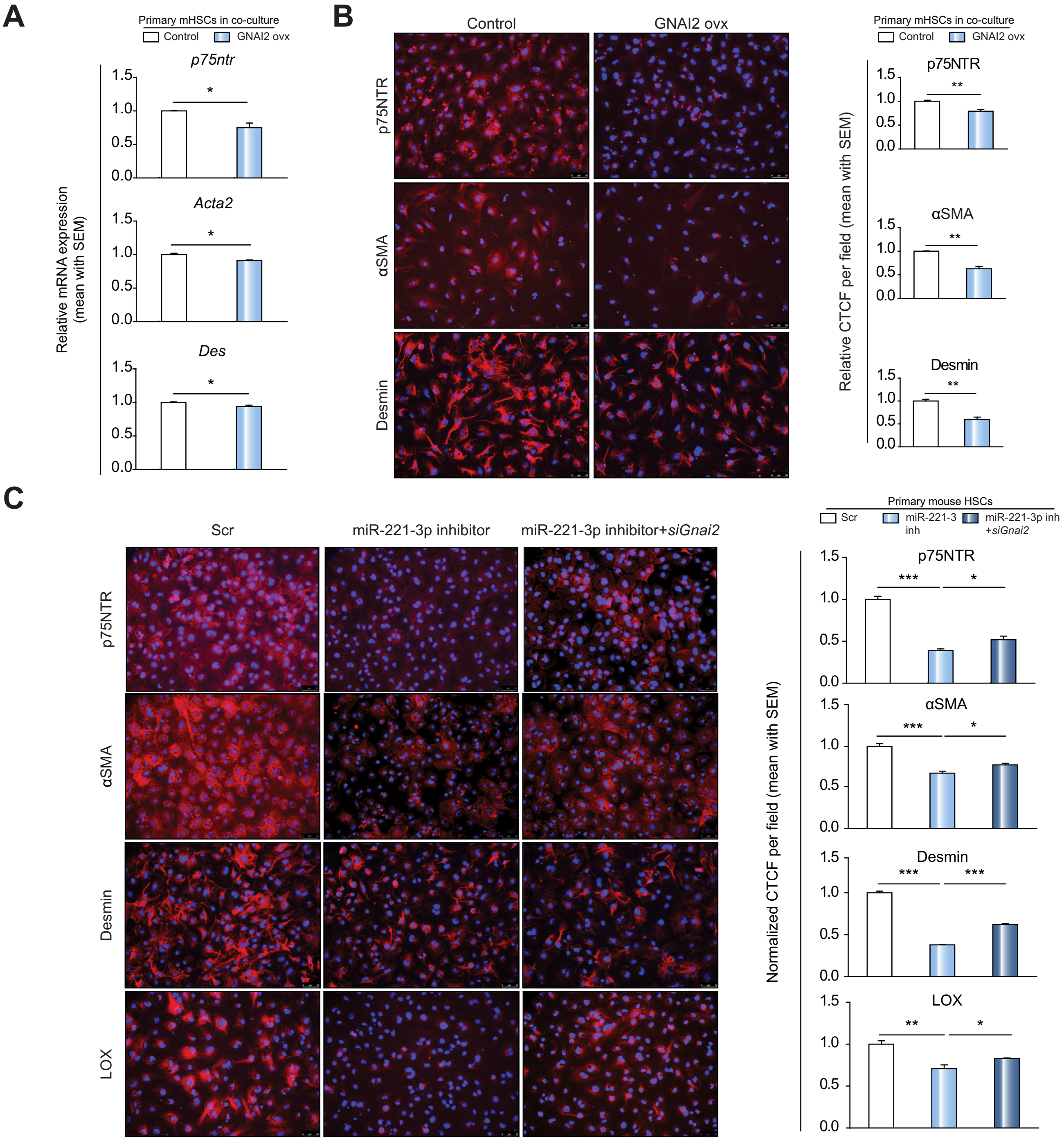

Fig. 5. MiR-221-3p inhibition in hepatocytes reduces HSC activation via GNAI2. Downregulation of *p75Ntr, Acta2,* and *Des* (A) mRNA (n = 3) and (B) protein levels in primary mouse HSCs co-cultured with GNAI2 overexpressing primary mouse hepatocytes *in vitro*. (C) Immunofluorescence staining showing derepression of HSC activation markers when primary mouse HSCs were co-cultured with primary mouse hepatocytes, which were co-transfected with miR-221-3p inhibitor and *Gnai2* siRNA. Magnification: 100×. Error bars represent ± SEM. One-way ANOVA was used for statistical evaluation. (**p* <0.05; ***p* <0.01; ****p* <0.001). CTCF, corrected total cell fluorescence (n ≥ 4 fields/group); mHSC, mouse hepatic stellate cell; Scr, scramble control; siRNA, small interfering RNA; SMA, smooth muscle actin.

which were co-transfected with miR-221-3p inhibitor and small interfering RNA (siRNA) against *Gnai2*. We found that the antifibrotic effects observed upon miR-221-3p inhibition were moderately, but significantly diminished when hepatocytes were co-transfected with *Gnai2* siRNA (Fig. 5C). These results suggest that GNAI2 indeed contributes to reduced HSC activation upon miR-221-3p inhibition in hepatocytes.

To address whether *Gnai2* overexpression affects functionality of hepatocytes, we analysed the expression of functional markers of hepatocytes such as *Alb, Krt18,* and *Hnf4a*. We found similar levels of all 3 hepatocyte markers upon *Gnai2* overexpression compared with control (Fig. S6B), indicating that overexpression of *Gnai2* does not affect hepatocyte functions.

We next sought to address the regulatory mechanism of HSC activation by GNAI2. Chemokine CCL2, one of the key regulators of inflammation and liver fibrosis,[30] has previously been reported to be elevated upon knockdown of *Gnai2* in mice.[31] Because we observed regulation of *Gnai2* by miR-221-3p, we reasoned that CCL2 could be a key component of miR-221-3p-GNAI2 signalling. We found a downregulation of *Ccl2* mRNA expression in hepatocytes, but not in non-parenchymal cells from mice with liver fibrosis injected with AAV TuD (Fig. 6A,B and Fig. S7). Consistent with these results, overexpression of GNAI2 in primary mouse hepatocytes led to a significant reduction in CCL2 secretion (Fig. 6C).

To confirm whether reduction in CCL2 is exerted by miR-221-3p inhibition, we collected supernatant from the culture medium of transfected primary mouse hepatocytes with miR-221-3p inhibitors. The ELISA results indeed confirmed reduction in secreted CCL2 levels (Fig. 6D). In accordance with these results, siRNA-based knockdown of *Gnai2* mRNA (Fig. 6E) led to a significant increase in secreted CCL2 levels (Fig. 6F).

To further verify the direct effects of CCL2 on HSCs, we treated primary mouse HSCs with 100 ng/ml CCL2 for 48 hours and measured expression of fibrotic markers by qPCR and immunofluorescence staining. The expression of *Col1a1*, *Acta2,* and *Tgfbr1* and protein levels of p75NTR, α-SMA, and desmin were significantly upregulated in primary mouse HSCs treated with CCL2 (Fig. 6G,H). Consistent with these results, primary mouse HSCs treated with the supernatant collected from primary mouse hepatocytes transfected with miR-221-3p inhibitors showed significant downregulation of *Col1a1*, *Acta2,* and *Tgfbr1* by qPCR and protein levels of p75NTR, α-SMA, and desmin (Fig. 6I,J). Taken together, our data suggest that upregulation of GNAI2 inhibited the expression of both *Ccl2* mRNA and CCL2 protein levels in hepatocytes that led to the inhibition of HSC activation and amelioration of liver fibrosis.

In addition to being a potent activator for HSC, CCL2 promotes chemotaxis for monocytes derived from bone marrow. We, therefore, investigated whether miR-221-3p mitigated fibrosis not only by reducing HSC activation but also by reducing monocyte recruitment. To address this, we analysed monocyte recruitment by fluorescence-activated cell sorting (Fig. S8A) as well as immunohistochemical analysis (Fig. S8B) for CD11b and F4/80. We observed lower number of CD11b and F4/80 cells, indicating that reduced monocyte recruitment indeed contributed to mitigation of liver fibrosis upon miR-221-3p inhibition in hepatocytes.

We next evaluated whether attenuation of fibrosis upon miR-221-3p inhibition is restricted to the CCl_4 model only. To answer this, we injected AAV TuD in mice fed with 3,5-di-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-containing diet, a model of cholestasis-induced fibrosis (Fig. 7A). We analysed fibrosis by histology, Sirius Red staining, hydroxyproline assay, fibrogenic markers' mRNA analyses in whole liver and isolated HSCs, and immunofluorescence staining for α-SMA, p75NTR, and desmin (Fig. 7B–F and Fig. S9A). The absence of dramatic changes in the expression of fibrogenic genes in isolated HSCs (Fig. 7F) might be due to cellular contamination resulting from the technically challenging perfusion of livers from DDC-fed mice (Fig. S9B). Taken together, as with the CCl_4 model, we observed attenuation of DDC-induced liver fibrosis upon miR-221-3p inhibition.

To examine whether our findings are applicable to human hepatocytes, we first inhibited miR-221-3p in primary human hepatocytes by transfecting them with miR-221-3p inhibitor, and collected medium for the next 48 hours (Fig. 8A). We then treated primary human myofibroblasts with culture medium collected from primary human hepatocytes (Fig. 8A). Subsequent qPCR analyses for *Col1a1*, *Acta2,* and *Tgfbr1* (Fig. 8B) and immunofluorescence staining for p75NTR, α-SMA, desmin, and LOX (Fig. 8C) showed significantly reduced expression of all markers in primary human myofibroblasts. These results indicate that culture medium collected from miR-221-3p-inhibited primary human hepatocytes is able to reduce activation of primary human myofibroblasts.

Finally, we investigated whether miR-221-3p expression is changed in primary human hepatocytes isolated from fibrotic livers. In concert with mouse data, miR-221-3p expression by qPCR analyses showed an upregulation in primary human hepatocytes isolated from fibrotic livers compared with non-fibrotic control livers (Fig. 8D). Furthermore, miR-221-3p upregulation correlated with reduced GNAI2 and concomitant upregulation of CCL2 protein levels in primary human hepatocytes obtained from fibrotic livers compared with respective controls (Fig. 8E).

Discussion

Current antifibrotic therapies mainly focus on HSCs such as the prevention of HSC activation, inhibition of fibrogenesis, and the resolution of fibrosis.[32] However, hepatocytes consist of up to 80% of the liver mass and are responsible for mediating a broad range of interactions between different cell types such as HSCs, Kupffer cells, and epithelial cells.[20] Our study highlights the therapeutic potential of targeting hepatocytes for treating liver fibrosis. We demonstrate that miR-221-3p is upregulated in hepatocytes during liver fibrosis. Importantly, *in vivo* knockdown of miR-221-3p by AAV TuD specifically in hepatocytes not only suppresses HSC activation but also alleviates CCl_4- and DDC-induced liver fibrosis in mice. Unlike other methods of RNA silencing that lead to systemic effects, hepatotrophic AAV TuD specifically targets hepatocytes and spares the off-target effects.[15,33,34] Therefore, as the most abundant cell type in the liver, the hepatocytes not only play an important role in regulating the activation of HSCs in liver damage, but also have an advantage of being a therapeutic target of liver fibrosis.

Mechanistically, we identified *Gnai2* as a novel miR-221-3p target. GNAI2, which belongs to the inhibitory G alpha family, is involved in signalling pathways related to cell cycle and hormone regulation.[35,36] GNAI2 is downregulated in HCC, and knockdown of GNAI2 expression significantly increases cell invasion and migration *in vitro*, suggesting that GNAI2 plays a role in suppressing metastasis.[26] Consistent with upregulation of miR-221-3p expression in a mouse model of liver fibrosis,

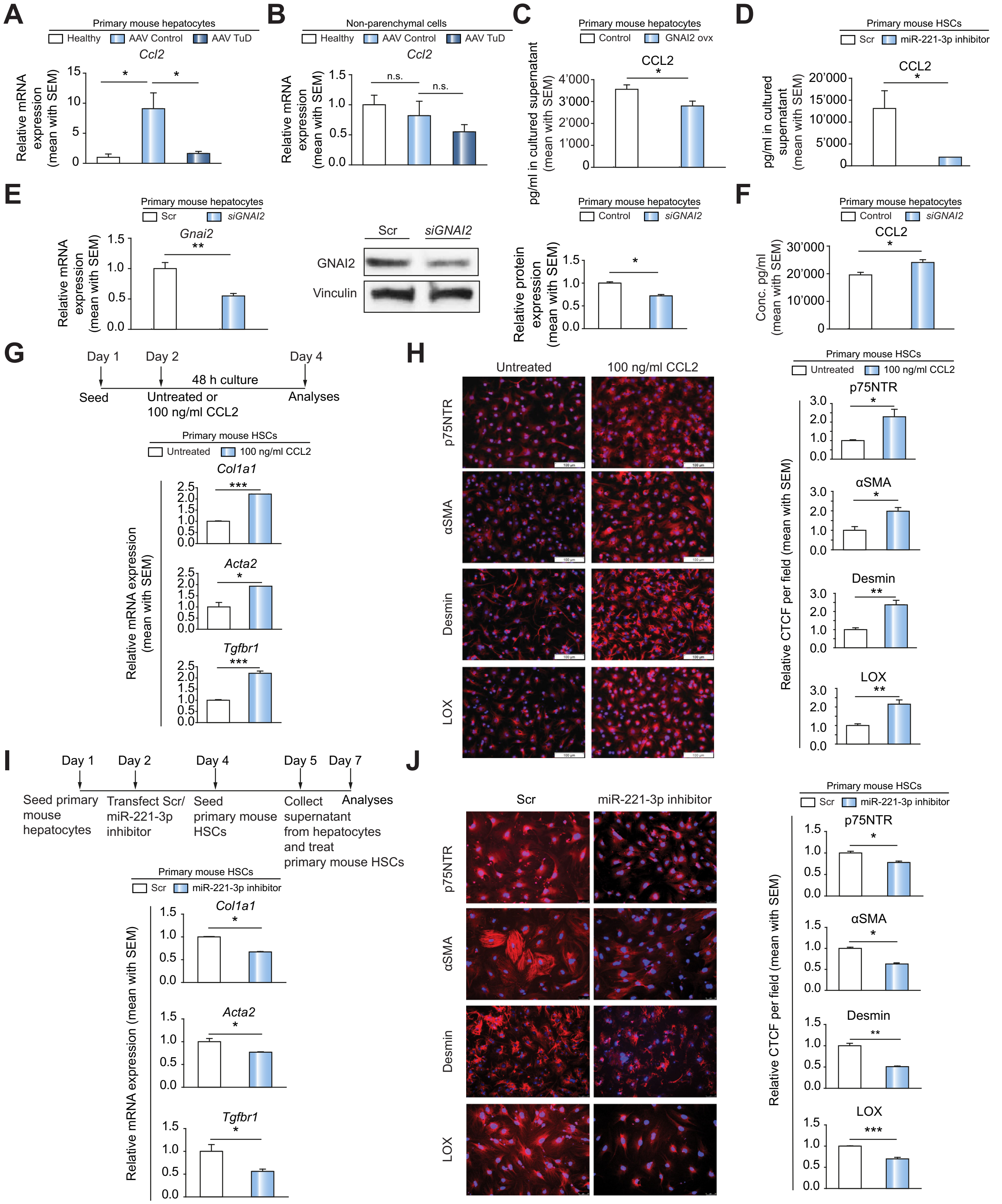

Fig. 6. CCL2 regulates the activation of HSCs. The mRNA expression of *Ccl2* is decreased in (A) primary mouse hepatocytes but not in (B) non-parenchymal cells *in vivo* (n = 7 mice/group). (C) Overexpression of GNAI2 or (D) downregulation of miR-221-3p lowered the CCL2 secretion in primary mouse hepatocytes *in vitro* (n = 9). (E) Knockdown of GNAI2 leads to (F) increased CCL2 secretion from hepatocytes (n = 3). (G) CCL2 increased levels of fibrotic markers, *Col1a1*, *Acta2*, and *Tgfbr1* (n = 4), and (H) protein expression of HSC activation markers, p75NTR, α-SMA, desmin, and LOX (n = 21 fields/group). (I) Reduced secretion of CCL2 upon treatment with miR-221-3p inhibitors facilitates suppression of fibrotic markers, *Col1a1*, *Acta2*, and *Tgfbr1* (n = 3) and (J) protein expression of HSC activation markers, p75NTR, α-SMA, desmin, and LOX (n = 15 fields/group). Magnification: 100×. Error bars represent ± SEM. One-way ANOVA was used for statistical evaluation. (*p <0.05; ***p <0.001; **p <0.01). AAV, adeno-associated virus; CTCF, corrected total cell fluorescence; HSC, hepatic stellate cell; Scr, scramble control; SMA, smooth muscle actin; TuD, Tough Decoy.

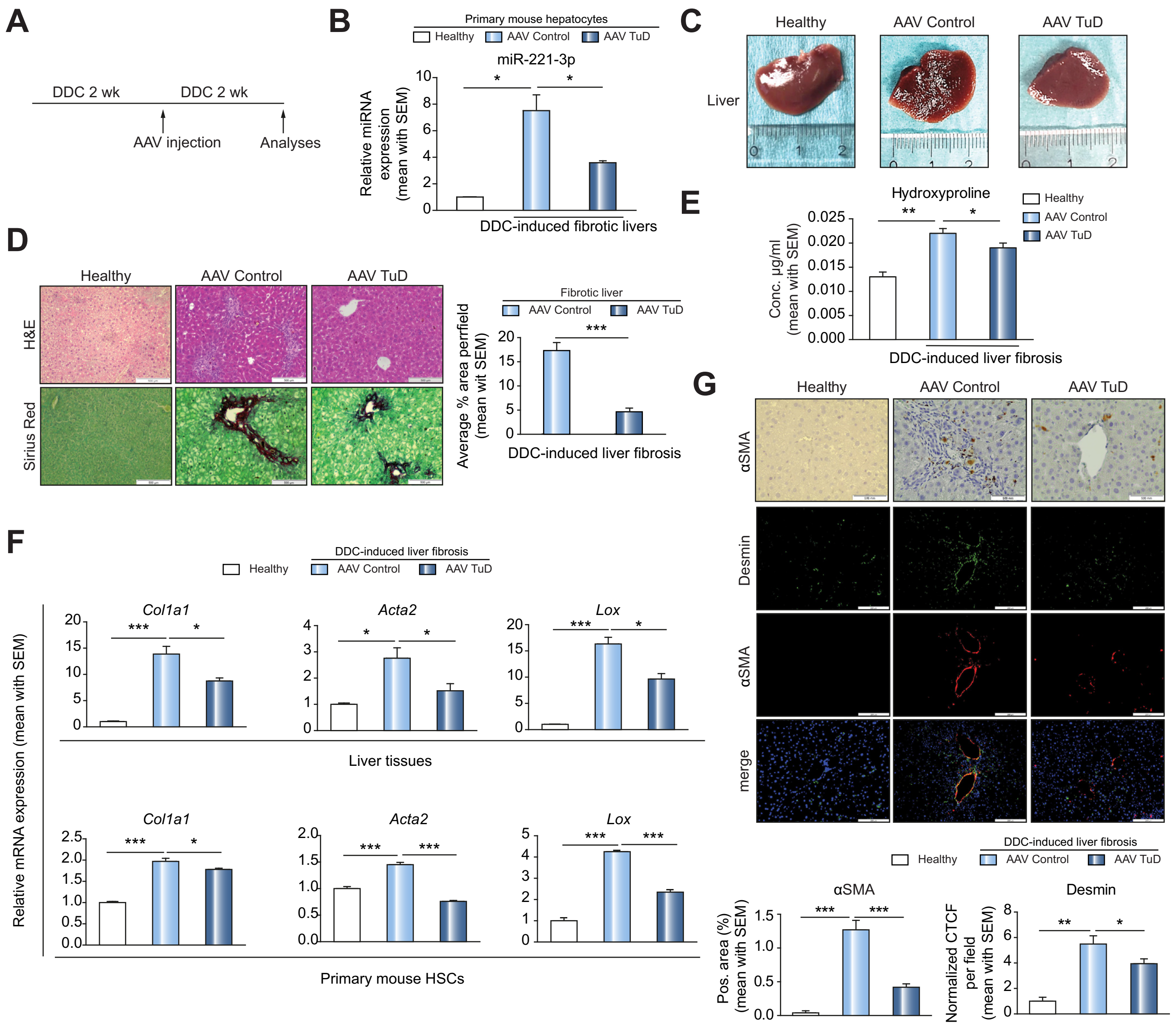

Fig. 7. Inhibition of miR-221-3p in hepatocytes mitigates DDC-induced liver fibrosis *in vivo*. (A) Schematic of DDC-induced liver fibrosis and time points of AAV injections. (B) qPCR result shows significant downregulation of miR-221-3p hepatocytes upon AAV TuD transfection (healthy n = 3 mice/group, AAV control n = 5 mice/group, AAV TuD n = 4 mice/group). (C and D) Suppression of liver fibrosis *in vivo* by AAV TuD as observed by less micronodules in gross morphology (C), less cellular infiltration (HE staining), and significantly reduced area of liver fibrosis shown by Sirius Red staining (D; n = 21 fields/group). (E) The amount of collagen significantly decreased in mice injected with AAV TuD. (F) qPCR shows that the expression of fibrotic markers *Col1a1*, *Acta2*, and *Lox* was significantly downregulated in both livers and primary mouse HSCs (healthy n = 3 mice/group, AAV control n = 5 mice/group, AAV TuD n = 4 mice/group). (G) Significant downregulation of α-SMA and desmin in fibrotic livers from mice injected with AAV TuD. The co-staining of α-SMA and desmin further revealed lower fibrosis in mice injected with AAV TuD. Magnification: 200× (α-SMA and desmin; n = 9 fields/group). Error bars represent ± SEM. One-way ANOVA was used for statistical evaluation. (*p <0.05; **p <0.01; ***p <0.001). AAV, adeno-associated virus; CTCF, corrected total cell fluorescence; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; HE, haematoxylin–eosin; HSC, hepatic stellate cell; miRNA, microRNA; qPCR, quantitative PCR; TuD, Tough Decoy.

GNAI2 levels are reduced in liver fibrosis, indicating its pathophysiological relevance. Importantly, GNAI2-overexpressing hepatocytes suppressed the HSC activation in co-culture experiments *in vitro*.

The CCR2–CCL2 signalling axis is a major regulator of HSC activation.[20] It has been shown that either blocking of CCL2 in rats or depletion of CCR2 in mice genetically[37] as well as pharmacological inhibition of CCL2 remarkably attenuates liver fibrosis.[38] Further, the high expression of CCL2 in HCC is associated with the poor prognosis of patients.[30] Furthermore, the loss of GNAI2 in *Gnai2*$^{-/-}$ mice leads to increased CCL2 in chronic inflammation.[31] Importantly, we found that overexpression of GNAI2 in primary mouse hepatocytes results in reduced levels of CCL2. Thus, our findings indicate that miR-221-3p inhibition in hepatocytes suppressed the activation of HSCs via regulation of the miR-221-GNAI2-CCL2 axis. Our data revealed that decreased Ccl2 levels upon miR-221-3p inhibition contribute to amelioration of fibrosis by not only reducing HSC activation but also lowering the monocyte recruitment in liver. We thus demonstrate for the first time that knocking down the expression of miR-221-3p in hepatocytes leads to decreased activation of HSCs, and finally amelioration of liver fibrosis.

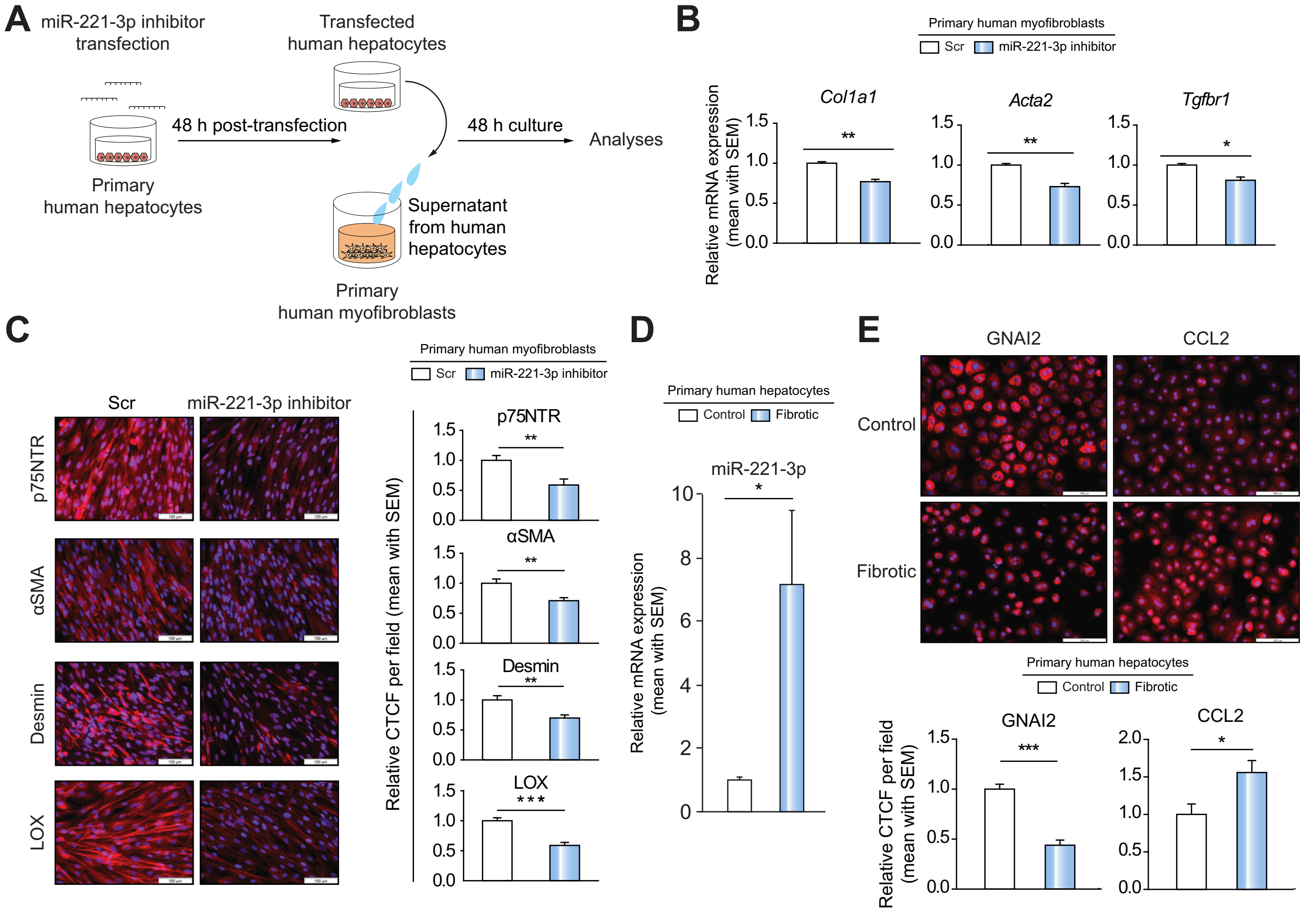

Fig. 8. Altered expression of miR-221-3p in primary human hepatocytes suppresses fibrogenic markers in primary human myofibroblasts. (A) Schematic of experimental design showing primary human hepatocytes transfected with miR-221-3p inhibitor. The primary human myofibroblasts were then exposed to the medium collected from miR-221-3p inhibitor-transfected primary human hepatocytes. (B) The qPCR analyses (n = 3) and (C) immunofluorescence staining for fibrogenic markers show reduced expression (n ≥ 6 fields/group). (D) Measurement of miR-221-3p expression by qPCR in primary human hepatocytes isolated from human fibrotic patients (n = 3) and controls (non-fibrotic livers, n = 3). (E) Immunofluorescence staining for GNAI2 and CCL2 on primary human hepatocytes collected on slides after cytospin. Magnification: 100×. Error bars represent ± SEM. One-way ANOVA was used for statistical evaluation. (*p <0.05; **p <0.01; ***p <0.001). CTCF, corrected total cell fluorescence; miRNA, microRNA; qPCR, quantitative polymerase chain reaction; Scr, scramble control; SMA, smooth muscle actin.

In concert with data obtained from mouse experiments, we demonstrated that miR-221-3p inhibition in primary human hepatocytes was capable of reducing fibrogenic markers in primary human myofibroblasts, indicating translational relevance of our findings. Although our *in vitro* experiments suggest a direct interaction between hepatocytes and myofibroblasts, there remains a possibility that protective activity of AAV TuD in hepatocytes also suppresses liver injury. In fact, the lower levels of ALT, AST, and bilirubin in mice injected with AAV TuD indicate less hepatocyte injury, which in turn may result in a reduced fibrosis response. It is important to mention that sustained overexpression of miR-221 for several months in mice promotes tumorigenicity in liver.[39] Furthermore, silencing of miR-221 by synthetic anti-miR is able to hinder hepatocellular carcinoma progression,[40] indicating reduced hepatocellular injury. Therefore, in light of our results and previously published data,[39,40] we cannot exclude the possibility that reduced injury of hepatocytes may have also contributed to the observed mitigation of fibrosis.

Taken together, we show that suppression of miR-221-3p in hepatocytes of fibrotic livers leads to faster resolution of the deposited extracellular matrix. Furthermore, downregulation of miR-221-3p induces the expression of GNAI2 in hepatocytes, which in turn inhibits CCL2 secretion, and hence leads to reduced HSC activation and amelioration of liver fibrosis. Our study highlights the promising potential of miRNA-221-3p as a therapeutic intervention for liver disease.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Authors' contributions

ADS conceived the idea, designed the study, and provided the conceptual framework for the study. H-CT performed all experiments, analysed data, and wrote the manuscript with the help of ADS. MO, AB, and SM further contributed with manuscript preparation. QY, AB, and MF helped with animal experiments. MK and AK performed *in situ* hybridisation. MPM provided conceptual evaluation of the project. All authors commented on the manuscript and declare no conflict of interest.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2018.12.016.

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Author names in bold designate shared co-first authorship

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