Therapeutic *HNF4A* mRNA attenuates liver fibrosis in a preclinical model

Graphical abstract

Highlights

- Restoration of HNF4A via mRNA delivery improves functions of fibrotic primary hepatocytes from both mice and humans.
- The mRNA encapsulated in lipid nanoparticles can be delivered into hepatocytes of the fibrotic liver.
- Lipid nanoparticle-mediated *HNF4A* mRNA delivery ameliorates fibrosis and cirrhosis in different chronic liver injury models.
- Paraoxonase 1, a therapeutic target of HNF4A, contributes to anti-fibrotic effects of HNF4A.
- *HNF4A* mRNA delivery affects macrophage infiltration and polarization as well as hepatic stellate cell activation.

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Lay summary

Liver fibrosis and cirrhosis remain unmet medical needs and contribute to high mortality worldwide. Herein, we take advantage of a promising therapeutic approach to treat liver fibrosis and cirrhosis. We demonstrate that restoration of a key gene, *HNF4A*, via mRNA encapsulated in lipid nanoparticles decreased injury in multiple mouse models of fibrosis and cirrhosis. Our study provides proof-of-concept that mRNA therapy is a promising strategy for reversing liver fibrosis and cirrhosis.

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Therapeutic HNF4A mRNA attenuates liver fibrosis in a preclinical model

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Background & Aims: Therapeutic targeting of injuries that require transient restoration of proteins by mRNA delivery is an attractive approach that, until recently, has remained poorly explored. In this study, we examined the therapeutic utility of mRNA delivery for liver fibrosis and cirrhosis. Specifically, we aimed to demonstrate the therapeutic efficacy of human hepatocyte nuclear factor alpha (HNF4A) mRNA in mouse models of fibrosis and cirrhosis.

Methods: We investigated restoration of hepatocyte functions by HNF4A mRNA transfection in vitro, and analyzed the attenuation of liver fibrosis and cirrhosis in multiple mouse models, by delivering hepatocyte-targeted biodegradable lipid nanoparticles (LNPs) encapsulating HNF4A mRNA. To identify potential mechanisms of action, we performed microarray-based gene expression profiling, single-cell RNA sequencing, and chromatin immunoprecipitation. We used primary liver cells and human liver buds for additional functional validation.

Results: Expression of HNF4A mRNA led to restoration of the metabolic activity of fibrotic primary murine and human hepatocytes in vitro. Repeated in vivo delivery of LNP-encapsulated HNF4A mRNA induced a robust inhibition of fibrogenesis in 4 independent mouse models of hepatotoxic- and cholestasis-induced liver fibrosis. Mechanistically, we discovered that paraoxonase 1 is a direct target of HNF4A and it contributes to HNF4A-mediated attenuation of liver fibrosis via modulation of liver macrophages and hepatic stellate cells.

Conclusion: Collectively, our findings provide the first direct preclinical evidence of the applicability of HNF4A mRNA therapeutics for the treatment of fibrosis in the liver.

Lay summary: Liver fibrosis and cirrhosis remain unmet medical needs and contribute to high mortality worldwide. Herein, we take advantage of a promising therapeutic approach to treat liver fibrosis and cirrhosis. We demonstrate that restoration of a key gene, HNF4A, via mRNA encapsulated in lipid nanoparticles decreased injury in multiple mouse models of fibrosis and cirrhosis. Our study provides proof-of-concept that mRNA therapy is a promising strategy for reversing liver fibrosis and cirrhosis.

Introduction

Transient protein expression by transfer of therapeutic mRNA considerably limits the risks encountered in viral gene therapy such as insertional mutagenesis, severe immune responses and potential activation of oncogenes. Tremendous progress has already been made with mRNA technology being applied to vaccine development1,2 and immuno-oncology.3 Lipid nanoparticle (LNP)-formulated mRNAs are shielded from humoral and cellular immunity and can be produced in large quantities at lower cost compared to viral vectors.4 mRNA therapeutics are particularly well-suited to treat injuries not requiring constant and life-long expression of therapeutic proteins, and offer the...
Fig. 1. HNF4A mRNA delivery restores function of fibrotic PHHs. (A) qPCR analyses of human HNF4A mRNA in patients with fibrosis: 0 (n = 9), 1 (n = 3), 2 (n = 3), 3 (n = 3) and 4 (n = 3) from MHH, Germany and 0 (n = 6), 2 (n = 4), 3 (n = 3), 4 (n = 5), 5 (n = 4), 6 (n = 5) from Zhongshan Hospital, China. (B) Immunofluorescence staining show reduced HNF4A protein expression in fibrotic PHHs. Scale bars, 100 μm. (C) Western blot and its quantification for wild-type (non-codon-
advantage over viral gene therapy of being adaptable to various medical conditions such as liver fibrosis and cirrhosis. The potential of this new class of mRNA-based drugs in liver fibrosis and cirrhosis, which contribute to millions of deaths annually and represent a major healthcare burden worldwide, remains to be harnessed.

To test whether mRNA delivery is able to mitigate liver fibrogenesis, we selected the human transcription factor HNF4A as a candidate for attenuation of liver fibrosis. The rationale for selecting HNF4A was based on its role as a master regulator of the hepatocyte phenotype and as a key factor in xenobiotic metabolism. Moreover, reduced expression of HNF4A has been reported in liver fibrosis and viral vector-mediated HNF4A restoration has previously been demonstrated to reverse features of chronic liver injury. Diminished HNF4A-dependent gene expression has been suggested as a major indicator of hepatocellular failure in alcoholic hepatitis. HNF4A, known to inhibit epithelial-to-mesenchymal transition, which is observed during wound healing and liver fibrosis, is downregulated in liver fibrosis in HCV-infected patients. Inhibition of the HNF4A transcriptional network is often seen with increased matrix stiffness in cirrhotic livers. Therefore, HNF4A has been suggested as a therapeutic target for the treatment of liver fibrosis. However, the question of whether an mRNA-based therapeutic coding for HNF4A is able to attenuate liver fibrosis and cirrhosis, needs to be addressed. Therefore, in our present study, we examined the therapeutic delivery of HNF4A mRNA in several mouse models of liver fibrosis and cirrhosis, and in human liver cells.

Materials and methods
Ethics Statement
RNA samples from fibrotic human livers were provided by our prospective biorepository after liver transplantation (Table S1A). The study was approved by the Ethics Committee of Hannover Medical School, Hannover, Germany (protocol number 933 for project Z2 of comprehensive research centre 738). Written informed consent was obtained from all participants. All experiments were performed in accordance with relevant guidelines and regulations. No organs or tissues were procured from prisoners. All liver transplantations of patients in this study were performed in Germany, where organ procurement and allocation are organized by Eurotransplant and the German Organ Transplantation Foundation. For the second cohort, qPCR analyses were provided by Shanghai Zhongshan Hospital, China (Table S1B) with ethical approval from the institute. There was no international transfer of tissue or RNA samples.

Statistical analyses
Statistical significance was determined with the 2-tailed Student's t test or 2-sided Welch's t test. Statistical significance was depicted with error bars representing ± SEM. p < 0.05 was considered significant.

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

Results
Human HNF4A mRNA restoration improves function of fibrotic hepatocytes
At first, we confirmed reduced endogenous HNF4A mRNA levels in patients with liver fibrosis, graded by the Ishak score (Fig. 1A). In 2 cohorts from Hannover Medical School, Germany, and Shanghai Zhongshan Hospital, China, liver tissues showed fibrosis stage-dependent reduction in HNF4A mRNA. Similarly, HNF4A protein expression was reduced in primary human hepatocytes (PHHs) from fibrotic human livers (Fig. 1B).

We then examined whether in vitro synthesized HNF4A mRNA produces functionally active HNF4A protein. We transacted wild-type (non-codon-optimized) or codon-optimized HNF4A mRNA into the human cervical carcinoma cell line (HeLa) cells as these lack endogenous HNF4A expression. Codon-optimized HNF4A mRNA-transfected cells showed higher HNF4A protein levels than those transfected with wild-type HNF4A mRNA (Fig. 1C). ZsGreen mRNA-transfected HeLa cells were our control. Subsequent transfection in PHHs confirmed functionally active codon-optimized mRNA as indicated by significant upregulation of HNF4A-target genes APOA2, APOB and TTR (Fig. 1D). We therefore used codon-optimized HNF4A mRNA for further experiments.

We determined whether HNF4A restores impaired functions in PHHs from fibrotic livers. Robust HNF4A protein expression in PHHs after transfection with HNF4A mRNA was observed (Figs. 1E-F). qPCR analyses showed significantly increased expression of hepatocyte markers albumin (ALB), alpha-1 antitrypsin (A1AT), transferrin (TF) and transthyretin (TTR) (Fig. 1G). Importantly, HNF4A mRNA upregulated expression of various enzymes involved in phase I and phase II drug metabolism and phase III drug transporters (Fig. 1H). HNF4A mRNA transfection in hepatocytes from non-fibrotic livers also enhanced expression of hepatocyte markers (Fig. 1G), enzymes, and transporters of drug metabolism (Fig. 1H). Significant increases in secreted protein levels of ALB and A1AT in PHHs transfected with HNF4A mRNA indicated that restoration of HNF4A in fibrotic PHHs revives function (Fig. 1I).

To examine drug responsiveness, we treated HNF4A mRNA-transfected PHHs with β-naphthoflavone, phenobarbital and rifampin and measured expression of CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4. Fibrotic PHHs had a reduced response to all drugs, and HNF4A mRNA delivery restored cytochrome activities significantly (Fig. 1J).

We next tested whether HNF4A mRNA delivery restores the function of fibrotic primary mouse hepatocytes (PMHs) isolated from mice with fibrosis due to carbon tetrachloride (CCL4) injections bi-weekly, for 8 weeks (Fig. S1A). qPCR analyses showed hepatocyte-specific expression of endogenous Hnf4a by qPCR (Fig. S1B) and HNF4A protein by co-staining with markers of different hepatic cells (Fig. S1C). Therefore, we aimed to restore HNF4A expression using hepatocyte-targeted LNPs. ZsGreen mRNA transfection led to robust corresponding protein expression in PMHs (Fig. S2A). HNF4A mRNA transfection induced expression of target genes in PMHs (Fig. S2B-D)
and led to functional restoration in fibrotic PMHs (Fig. S2E-K). Of note, DNASTAR MegAlign shows 95.8% sequence homology between human HNF4A and mouse Hnf4a. DNA binding domains of both exhibit similar molecular function due to high homology. Thus, our in vitro analyses confirm human HNF4A mRNA delivered into PMHs improves function, as observed in PHHs.

**Targeted delivery of LNP-encapsulated mRNA into hepatocytes of fibrotic livers**

We established efficient LNP-mediated mRNA delivery into hepatocytes of murine fibrotic livers. LNPs direct cell entry by interacting with apolipoprotein E, and are subsequently internalized into hepatocytes via endocytosis.18–21 However, whether LNP-formulated mRNA reaches hepatocytes of fibrotic livers efficiently has remained unknown. LNP carrying mRNA encoding for *Photinus pyralis* luciferase (Luc/LNP) or ZsGreen (ZsGreen/LNP) were injected into CCl4-induced fibrotic and non-fibrotic control (wild-type) mice i.v. (Fig. 2A). In vivo imaging revealed robust bioluminescence exclusively in the liver from 8 hours to 120 hours after Luc/LNP injection in both groups (Fig. 2B). Co-localization studies showed robust ZsGreen protein expression specifically in hepatocytes of fibrotic and control mice, but not in other hepatic cells (Fig. 2C). Our results demonstrated that LNP-formulated mRNA technology could specifically and efficiently target hepatocytes in murine fibrotic livers.

Successful mRNA delivery should be therapeutic but with negligible immune response. Therefore, we first monitored immune response after injecting different doses of ZsGreen/LNP (Fig. S3A). Measurement of different cytokines and chemokines indicated that mRNA doses of 1 mg/kg and 2 mg/kg did not elicit substantial immune responses, whereas 4 mg/kg mRNA induced a significant immune response (Fig. S3B). Therefore, we used 2 mg/kg mRNA/LNP for subsequent experiments.

We next examined whether repeated i.v. administration of 2 mg/kg mRNA/LNP was safe in fibrotic mice (Fig. S4A). Repeated injections of 2 mg/kg LNP encapsulating *HNF4A* mRNA (henceforth referred to as *HNF4A*/LNP) every 5 days induced no significant elevations of most cytokines and chemokines (Fig. S4B). Thus, administration of either single or multiple doses of 2 mg/kg *HNF4A*/LNP is well tolerated.

**Delivery of human *HNF4A* mRNA attenuates toxin- and cholestasis-induced fibrosis**

To address whether systemic administration of *HNF4A* mRNA inhibits liver fibrosis, we used mice with toxin- (repeated CCl4 injection) (Fig. 3A-H) or cholestasis-induced fibrosis (induced via 3,5-diethoxycarbonyl-1,4-dihydrocollidine [DDC]-containing...
Fig. 3. Therapeutic HNF4A/LNP delivery inhibits toxin- and cholestasis-induced liver fibrosis. (A, I) Schematic of experiments analysing effect of human HNF4A mRNA delivery in mouse models of CCl4- (A-H) and DDC (I-P)-induced liver fibrosis (n = 6 mice per CCl4 model, n = 4 mice per DDC model). (B, J) Hnf4a qPCRs after CCl4 injection (B) or after DDC diet (J). (C, K) Western blots show HNF4A protein expression. (D, L) Liver function tests for ALT and bilirubin show reduced injury upon HNF4A/LNP administration. (E, M) Hydroxyproline assay. (F, N) Immunohistochemical images of H&E, desmin, and Sirius red stainings. Scale bars, 100 μm. (G) Quantification of Sirius red and desmin stainings. (H, P) qPCRs for Col1a1, Col2a1, and Acta2. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ALT, alanine aminotransferase; CCl4, carbon tetrachloride; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; LNP, lipid nanoparticle; P, postnatal day; WT, wild-type.
HNF4A mRNA levels significantly decreased in fibrotic livers from both models (Fig. 3B, 3J). HNF4A/LNP or ZsGreen/LNP (henceforth referred as control) were injected i.v. into fibrotic mice at 2 mg/kg per injection (Fig. 3A, 3I). HNF4A protein expression was confirmed in HNF4A/LNP-injected mouse livers (Fig. 3C, 3K) and was absent in control mice, since the HNF4A antibody was only specific to human but not mouse HNF4A. HNF4A/LNP-injected mice showed significantly reduced collagen, suggesting decreased fibrosis in both CCl4 (Fig. 3E) and DDC (Fig. 3M) models. Histological analyses, desmin and Sirius red staining further confirmed reduced fibrosis in CCl4 (Fig. 3F-G) and DDC (Fig. 3N-O). Additionally, qPCR showed significantly decreased expression of fibrogenic marker genes, Col1α1, Col2α1 and Acta2 in (Fig. 3H, P). Thus, our data together provide evidence that HNF4A mRNA delivery attenuates fibrosis in mouse models of toxin- as well as cholestasis-induced fibrosis.

We compared the efficacy of HNF4A/LNP with recombinant adeno-associated virus (AAV) vector serotype 8, by injecting fibrotic mice with 1x10^11 AAV8 particles, encoding optimized human HNF4A under the transthyretin promoter. Liver function test, hydroxyproline assay, histological analyses, desmin and Sirius red staining, and qPCR analyses of fibrogenic genes showed attenuation of liver fibrosis by HNF4A/LNP is comparable to AAV-mediated HNF4A delivery in CCl4 (Fig. 3D-H) or DDC-induced fibrosis (Fig. 3L-P).

We then investigated whether transient delivery of HNF4A/LNP restores endogenous *Hnf4a* expression. qPCR analyses showed that HNF4A/LNP significantly increased endogenous mouse *Hnf4a* expression (Fig. 5A, B), indicating a long-lasting phenotypic change in hepatocytes. We further investigated whether human HNF4A mRNA delivery enhances the expression of other hepatic transcription factors, which can restore endogenous mouse *Hnf4a*. We transfected fibrotic PMHs with human HNF4A mRNA and determined the expression of mouse *Hnf1a* via qPCR and western blot analyses (Fig. S6A). *Hnf1a* could further induce endogenous Hnf4a expression via binding to its promoter region. Within 24 hours after human HNF4A mRNA transfection, we observed increased expression of *Hnf1a* mRNA (Fig. S6B) and protein in hepatocytes (Fig. S6C), and of endogenous mouse *Hnf4a* (Fig. S6D).

To determine whether human HNF4A mRNA influences stability of mouse endogenous Hnf4a mRNA, we analyzed mRNA decay as a measure of mRNA stability following transcriptional inhibition with Actinomycin D treatment in PMHs (Fig. S6E). We
found that Actinomycin D caused similar decay of mouse Hnf4a mRNA in cells transfected with either human HNF4A mRNA or control ZsGreen mRNA (Fig. S6F). These results support our findings that human HNF4A mRNA favors transcription of endogenous mouse Hnf4a, in part via Hnf1a, without affecting mRNA stability (Fig. S6G).

We addressed whether alternate HNF4A/LNP-treatment schedules (2 weeks or 6 weeks) and doses (0.5 and 1 mg/kg) are equally effective. Although both additional treatment schedules (2 weeks or 6 weeks) and doses (0.5 and 1 mg/kg) are equally effective. Although both additional treatment schedules (2 weeks or 6 weeks) and doses (0.5 and 1 mg/kg) are significantly reduced liver fibrosis (Fig. S7). Of the 2 additional doses, only 1 mg/kg significantly reduced liver fibrosis (Fig. S8). Together, these results indicate that dose and schedule optimization may increase effectiveness of HNF4A mRNA delivery on liver fibrosis attenuation.

We next examined whether HNF4A mRNA reduces acute hepatocyte injury induced by either CCl4 administration or bile duct ligation (Fig. S9A). Significant reduction in endogenous Hnf4a in hepatocytes of mice with either acute CCl4 injury or 7 days after bile duct ligation, was observed (Fig. S9B). We performed H&E, TUNEL, Ki67, CD45 and F4/80 staining (Fig. S9C-E).

Delivery of human HNF4A mRNA inhibits liver injury in a mouse model with features of cirrhosis

To determine whether HNF4A mRNA inhibits cirrhosis in mice injected with CCl4 twice weekly for 16 weeks (Fig. 4A), HNF4A/LNP or ZsGreen/LNP were administered i.v. starting from 12 weeks, once every 5 days. Cirrhotic mice showed loss of Hnf4a (Fig. 4B). HNF4A/LNP delivery led to HNF4A protein expression, as determined by western blot analyses (Fig. 4C). Injury was evaluated by measurement of ALT, hydroxyproline content, body weight changes, activity score, histology, desmin, Sirius red staining and qPCR analyses for fibrogenic genes. All these analyses provided evidence that HNF4A/LNP inhibits cirrhosis in mice (Fig. 4D-J).
Fig. 6. HNF4A reduces fibrosis by directly targeting the paraoxonase 1 gene. (A) Heat map shows significantly deregulated genes. (B) Selected genes encoding secretory proteins between Control (ZsGreen/LNP) and HNF4A/LNP treatment groups. (C) KEGG pathway; GO molecular function and GO biological process enrichment plot. (D) HNF4A binding site prediction within the genomic upstream promoter region of paraoxonase 1. (E-H) qPCRs and gel confirmation in ZsGreen or HNF4A mRNA-transfected (E, F) PMHS, Hepa1-6, and (G, H) 3T3 cells, after ChIP with 3 different primer pairs surrounding the potential HNF4A BS. Values are normalized to input control for HNF4A antibody and IgG control ± SEM (n = 3). (I) Schematic of experiment to verify HNF4A mRNA's effect on paraoxonase 1. (J) qPCR for paraoxonase 1 expression, (K) dynamic paraoxonase 1 activity, and (L) average paraoxonase 1 activity (n = 3). BS, binding site; ChIP, chromatin immunoprecipitation; GO, gene ontology; LNP, lipid nanoparticle; PMHS, primary mouse hepatocytes.
Fig. 7. HNF4A-Pon1 pathway induces macrophage polarization and deactivation of HSCs. (A) Schematic of co-culture experiments. (B) qPCR for paraoxonase 1 in PMHs. (C) ELISA showing secreted CCl2 levels in PMHs. (D) FACS analysis of M1- or M2-positive macrophage distribution pattern, (E) quantification of M1/M2 percentage, and (F) qPCRs for M1 (iNOS and IL6) and M2 (Arg1 and Fizz1) related genes in macrophages 48 h after culturing in supernatant from ZsGreen mRNA, HNF4A mRNA, and, HNF4A mRNA and paraoxonase 1 siRNA co-transfected PMHs. (G) Immunofluorescence stainings for desmin, a-SMA, and LOX (H) quantified corrected total cell fluorescence after co-culture experiment. (I) qPCRs for Acta2, Colta1, and Col2a1 (n = 3). HSCs, hepatic stellate cells; KCs, Kupffer cells; PMHs, primary mouse hepatocytes; siRNA, small-interfering RNA.
Fig. 8. Single-cell RNA sequencing confirms HNF4A/LNP treatment reduces macrophage infiltration and HSC activation. (A) Schematic overview of designed scRNA-seq experiment for hepatocytes and non-parenchymal cells after ZsGreen/LNP and HNF4A/LNP treatment in high-dose CCl4-injected mice. (B) UMAP of Leuven clustering of cells from control and HNF4A groups. (C) Dotplot of cluster marker genes. Radii of circles are proportional to number of cells expressing a
Human HNF4A mRNA delivery suppresses cholestasis and fibrosis in Mdr2−/− mice

Severe forms of cholestasis are frequent causes of cirrhosis.22 Hence, we addressed whether HNF4A mRNA suppresses injury in multidrug resistance gene 2 knockout mouse (Mdr2−/−), a surrogate mouse model of progressive familial intrahepatic cholestasis.23,24 We injected 12–week old Mdr2−/− mice with 2 mg/kg HNF4A/LNP or ZsGreen/LNP, once every 5 days, before sacrificing them at 16 weeks of age (Fig. 5A). We observed lower expression of Hnf4a mRNA in Mdr2−/− mice compared to age-matched FVB background control mice (Fig. 5B). HNF4A/LNP-injected mice showed HNF4A protein expression (Fig. 5C), significantly decreased levels of bilirubin, hydroxyproline content, reduced expression of Ck19, Sox9 and Epcam, reduced injury by histology, desmin and Sirius red staining, and decreased expression of fibrogenic genes, Col1a1, Col2a1 and Acta2 (Fig. 5D–I). Thus, our experiments in Mdr2−/− mice demonstrate that HNF4A mRNA delivery attenuates features of severe cholestasis in mice.

Mechanisms of HNF4A mRNA-mediated inhibition of fibrogenesis: paraoxonase 1 contributes to anti-biofilm effects of HNF4A mRNA

To elucidate mechanisms of HNF4A mRNA-mediated fibrosis inhibition, we performed transcriptome analyses on cirrhotic livers from HNF4A/LNP- and control ZsGreen/LNP-treated mice. We found 67 genes with significant changes in expression (p <0.01; Fig. 6A). We examined if HNF4A mRNA delivery in hepatocytes influences other hepatic cells via altered gene expression of secretory proteins. Thus, we selected 13 significantly deregulated secreted protein-encoding genes (Fig. 6B). Gene ontology and KEGG pathway analyses indicated enrichment of drug and glutathione metabolism pathways (Fig. 6C). To identify direct targets of HNF4A, among these 13 genes, we used the TRANSFAC transcription factor database, and found paraoxonase (Pon1) has 4 binding sites for HNF4A in its promoter region (Fig. 6D). Secreted protein PON1, primarily produced by hepatocytes, has an important role in the antioxidant system and a hepatoprotective function in liver injury.25 Chromatin immunoprecipitation and qPCR confirmed binding of HNF4A with the Pon1 promoter in PMHs and Hepa 1-6 cells (Fig. 6E,F), as well as with the mouse Pon1 promoter, in murine 3T3 cells that lack endogenous Hnf4a expression (Fig. 6G,H). Further, we confirmed elevation in Pon1 mRNA levels, and enhanced PON1 activity upon transfection of PMHs with human HNF4A mRNA (Fig. 6I–L). We also confirmed increased Pon1 expression upon HNF4A mRNA delivery in 4 models of chronic liver injury (CCL4-induced fibrosis and cirrhosis, Mdr2−/−, and DDC-induced cholestasis) (Fig. S10). Thus, we identified the Pon1 promoter as a direct target of HNF4A and demonstrated that expression and activity of Pon1 was enhanced after delivery of HNF4A mRNA, in vitro and in vivo.

Human liver bud model confirms that PON1 contributes to HNF4A-mediated attenuation of fibrogenesis

We next established a human liver bud model (Fig. S11A,B), generated by aggregation of 4 hepatic cell types: functional PHH- derived organoids, human Kupffer cells (transduced with a GFP-encoding lentiviral vector), primary human hepatic stellate cells (HSCs: transduced with a dsRed-encoding lentiviral vector) and endothelial cells. Transfection of HNF4A mRNA in hepatocyte organoids reduced secreted levels of C-C motif chemokine ligand 2 (CCL2) and tissue inhibitor of metalloproteinase 1 (TIMP1) (Fig. S11C) in the liver bud and decreased numbers of Kupffer cells, as indicated by reduced GFP fluorescence (Fig. S11B). Importantly, expression of fibrotic markers ACTA2, COL1A1 and LOX also decreased (Fig. S11D). Inhibition of PON1 by small-interfering RNA (siRNA) diminished the anti-biofilm effects observed upon HNF4A mRNA transfection. These results confirmed our findings that PON1 contributes to HNF4A-mediated attenuation of fibrogenesis in human cells as well.

HNF4A-Pon1-mediated reduction in macrophage infiltration and modulation of M1 to M2 polarization contributes to reduced fibrogenesis

To further verify if PON1 contributes to HNF4A-mediated inhibition of fibrogenesis, we co-transfected PMHs with HNF4A mRNA and siRNA against Pon1 (Fig. 7A). Pon1 siRNA-transfected PMHs showed robust Pon1 knockdown (Fig. 7B). 48 hours after transfection, we collected cell culture medium and examined its effect on Kupffer cells and HSCs (Fig. 7A). Pon1 has been reported to degrade CCL2,26 a pro-inflammatory chemokine and a critical regulator of liver fibrosis.27 We confirmed strongly suppressed CCL2 secretion in medium collected from HNF4A mRNA-transfected PMHs (Fig. 7C). Medium from cells transfected with only HNF4A mRNA increased the number of M2 macrophages, which are considered as anti-inflammatory macrophages (Fig. 7D,E). Of note, transition into M2 macrophages has been shown to suppress fibrosis in mice.28 Medium collected from PMHs co-transfected with Pon1 siRNA and HNF4A mRNA, induced the M1 phenotype (Fig. 7D,E). Gene expression analyses...
of IL6, iNOS, Arg1 and Fizz1 confirmed that HNF4A drives macrophages into the M2 phenotype, whereas inhibition of Pon1 suppressed transition into M2 macrophages (Fig. 7F). Likewise, exposure of HSCs to medium collected from PMHs transfected with HNF4A mRNA reduced mRNA and protein levels of fibrogenic markers such as desmin, αSMA, LOX (Fig. 7G-I). Further, the co-transfection of PMHs with Pon1 siRNA and HNF4A mRNA diminished the HNF4A-mediated reduction in HSC activation (Fig. 7G-I). Thus, the HNF4A-Pon1 pathway suppresses fibrogenesis in HSCs and induces transition of macrophages into the anti-inflammatory macrophage phenotype, which together contribute to the inhibition of fibrogenesis.

To confirm the effect of HNF4A mRNA delivery in chronic liver injury models, we analyzed M1 and M2 macrophages, and pro-fibrogenic markers in established chronic liver injury models. qPCR analysis showed decreased expression of Tnfa, Ccl2 and iNos, and increased expression of Arg1 and Fizz after HNF4A mRNA/LNP treatment in all 4 chronic liver injury models (Fig. S12A,C,E,G). F4/80 immunohistochemistry staining indicated reduced macrophage infiltration after HNF4A mRNA/LNP treatment (Fig. S12B,D,F,H). Further, expression of pro-fibrogenic genes Tgfb1, Pdgf, and Timp1, and protein levels of αSMA decreased after HNF4A mRNA/LNP treatment (Fig. S12).

### Single-cell RNA sequencing analyses upon HNF4A mRNA delivery

To gain further insights into the molecular and cellular changes induced by HNF4A, we first injected mice with a single administration of CCL4 (8 μl/g of 10% CCL4), thus inducing acute liver injury. 24 hours later, we treated mice once with HNF4A/LNP or with ZsGreen/LNP (Fig. 8A). We chose the acute injury model rather than chronically developed liver fibrosis since this enabled the identification of direct targets of HNF4A. 24 hours after HNF4A/LNP injection, we isolated all liver cells by Percoll-density gradient centrifugation and subjected them to 10x Genomics single-cell RNA sequencing (scRNA-seq). Our scRNA-seq analyses, performed on 13,880 liver cells, identified different populations of liver cells, including hepatocytes, cholangiocytes, HSCs, macrophages, Kupffer cells, neutrophils, LSECs, dendritic cells, plasma B-cells and T-cells (Fig. 8B,C). Hepatocytes and macrophages showed substantial transcriptomic differences (Fig. 8D,E); HNF4A/LNP-injected mice showed decreased expression of pan-macrophage marker CD68 (Fig. 8F). MacSpectrum computational analysis of scRNA-seq data was reported to define macrophage polarization.29 Our MacSpectrum analyses suggested transition into M2-macrophages (Fig. 8G-I). Additionally, we observed decreased expression of pro-inflammatory M1 marker Ccl2 and increased anti-inflammatory M2 marker Arg1 (Fig. 8K,L) in HNF4A/LNP-injected mice. We observed increased expression of hepatocyte genes Apoa2, Mup3, Mup20, Fah, Cyp2a12 and Cyp3a11 in the hepatocyte cluster, (Fig. 8M,N) and decreased expression of fibrotic genes Tagln, C3, Furin, Egr1 and Klf2 in the HSC cluster (Fig. 8O,P). Together, scRNA-seq analyses confirmed our findings that HNF4A mRNA delivery attenuates liver fibrosis by modulating expression of genes in cellular compartments including hepatocytes, macrophages and HSCs.

### Discussion

Taken together, our work provides the first evidence that therapeutic mRNA delivery restores intracellular HNF4A transcription factor levels, induces endogenous Hnf4a expression, and improves metabolic activity of targeted hepatocytes. Our data show that hepatocyte-specific delivery of HNF4A mRNA in injured livers attenuates fibrosis and cirrhosis in multiple independent mouse models of liver diseases.

Our study identified a new mechanism showing restoration of HNF4A modulates non-parenchymal cells such as macrophages and HSCs. Our transcriptome analyses of the whole liver and scRNA-seq led to identification of Pon1 as a novel direct target of HNF4A. Increased Pon1 promoted an anti-inflammatory M2 phenotype in macrophages and suppressed the pro-fibrogenic activity of HSCs. However, the role of macrophages in chronic liver diseases is complex, with the emergence of a new macrophage phenotype in the fibrotic liver of rodents and human samples.31 Thus, further classification and evaluation of macrophage functions after HNF4A mRNA/LNP treatment are required to understand how HNF4A mediates its anti-fibrotic effect through macrophage modulation. Other unknown mechanisms may further contribute to the anti-fibrotic properties of HNF4A.

How does human HNF4A mRNA restore expression of mouse Hnf4a in the liver? Exogenous human HNF4A mRNA may increase stability of endogenous mouse Hnf4a. However, our results from mRNA stability analyses excluded this. Exploring another possibility that exogenous human HNF4A may bind to and enhance expression of other mouse transcription factors, we found HNF4A mRNA delivery increases mouse Hnf1a expression, which can bind to promoter regions of multiple hepatocyte genes, including Hnf4a. Therefore, we speculate that using a feedback loop mechanism, exogenous human HNF4A first induces endogenous Hnf1a expression, which in turn binds to the endogenous Hnf4a promoter, thus restoring mouse Hnf4a levels.

A major strength of our study is the lack of any adverse immune response due to mRNA or LNP following repeated HNF4A mRNA administration, even though all in vivo experiments were performed with immunocompetent mice. This is particularly important in case of fibrosis relapse, frequently observed in patients. Multiple injections of viral vectors may not be feasible due to development of viral capsid-specific antibodies after the first injection and, hence, may preclude repeated use in case of fibrosis relapse and cirrhosis. Another strength of HNF4A mRNA delivery is that despite a short half-life of delivered mRNA, it led to cyclic expression of endogenous Hnf4a. Therefore, this may be a safer therapeutic approach than continuous expression via viral vectors. Additionally, using LNP-mediated mRNA delivery enables large-scale manufacturing at low costs, within a short duration. Another relevant factor in our study is that we achieved attenuation of injury in fibrotic and cirrhotic mice using human HNF4A mRNA. This suggests human HNF4A mRNA is suitable to exert anti-fibrotic effects in vivo. However, it is possible that these anti-fibrotic effects could be more robust when tested in patients with fibrosis. Our in vitro experiments demonstrating restoration of lost functions in fibrotic PHHs, and the robust anti-fibrotic effects in the human liver bud after human HNF4A mRNA delivery are encouraging and highlight the potential of our findings for future clinical translation.

HNF4A may regulate expression of multiple CYP enzymes, thereby influencing liver damage induced by toxins such as CCl4 and DDC. We indeed observed increased expression of multiple CYP enzymes. However, this in principle should aggravate, rather than mitigate toxin-induced damage. Activities of CYP enzymes...
are essential to generate the trichloromethyl radical leading to subsequent liver damage. The absence of a few CYP enzymes have been reported to protect mice against CCL4-induced fibrosis; for example, Cyp2e1−/− mice are resistant to CCL4-induced hepatoxocity.22 More recently, loss of Cyp2a5 was shown not to affect CCL4-induced liver fibrosis.23 Similarly, reduced levels of Cyp3a enzymes, which are required for DDC metabolism, can suppress DDC-induced liver fibrosis.24 Therefore, it is unlikely that mice showing amelioration of liver fibrosis upon HNF4A mRNA treatment were protected from the toxin itself.

In summary, our findings provide the first preclinical evidence that therapeutic HNF4A mRNA delivery via LNP attenuates liver fibrosis and cirrhosis. Our study may serve as a novel paradigm for application of mRNA-based therapeutics for the treatment of liver fibrosis.

Abbreviations
A1AT, alpha-1 antitrypsin; AAV, adeno-associated virus; ALB, albumin; ALT, alanine aminotransferase; CCL2, chemokine C-C motif ligand 2; CCL4, carbon tetrachloride; DC, dendritic cells; DDC, 3,5-bumin; ALT, alanine aminotransferase; CCL2, chemokine C-C motif ligand 2; HNF4A, hepatocyte nuclear factor 4 alpha; HSC, hepatic stellate cell; LSECs, liver sinusoidal endothelial cells; Mdr2, multidrug resistance gene 2; MPI, macrophage polarization index; PHHs, primary human hepatocytes; PMHs, primary mouse hepatocytes; PON1, paraoxonase 1; scRNA-seq, single-cell RNA sequencing; siRNA, small-interfering RNA; TIMP1, tissue inhibitor of metalloproteinase 1.

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Conflict of interest
MP, NH and FC are employees of CureVac AG, Tuebingen Germany, a publicly listed company developing mRNA-based vaccines, cancer immunotherapeutics and mRNA-based protein replacement therapies. All authors may hold shares or stock options in the company. MP, NH and FC are inventors on several patents on mRNA-related technology and use thereof. Other authors declare no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors’ contributions
ADS conceived the idea, designed the study and provided the conceptual framework for the study. TY, RK, QH, ZD and RL performed all experiments and analyzed the data. MP, NH and FC provided the mRNA and LNP. QYu assisted during animal experiments. CF contributed to measurement of cytokines and chemokines. FWR V provided primary human hepatocytes. ADS wrote the manuscript with the help of TY, MP, MO, FC, and AB. RT, BE and EJ provided RNA samples from MHH cohort. GS, QY and XS helped with the collection and analyses of HNF4A mRNA from Zhongshan Hospital cohort. AB helped with the in silico analyses. DG and MJA-B performed the computational analysis of the scRNA-seq data. MP, AB, AS, AV, NH, FC, TC and MO provided conceptual evaluation of the project. All authors commented on and approved the manuscript.

Data availability statement
The authors declare that data supporting the findings of this study are available within the article and its supplementary information files. The scRNA sequencing data and microarray data generated in this study have been deposited in NCBI’s Gene expression Omnibus and are accessible through GEO Series accession number GSE165277.

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