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Permalink
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Publication Date
2017-06-01

DOI
10.1016/j.omtn.2017.04.014

Peer reviewed
Identification of Novel Fibrosis Modifiers by In Vivo siRNA Silencing

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Fibrotic diseases contribute to 45% of deaths in the industrialized world, and therefore a better understanding of the pathophysiological mechanisms underlying tissue fibrosis is sorely needed. We aimed to identify novel modifiers of tissue fibrosis expressed by myofibroblasts and their progenitors in their disease microenvironment through RNA silencing in vivo. We leveraged novel biology, targeting genes upregulated during liver and kidney fibrosis in this cell lineage, and employed small interfering RNA (siRNA)-formulated lipid nanoparticles technology to silence these genes in carbon-tetrachloride-induced liver fibrosis in mice. We identified five genes, Egr2, Atpl1a2, Fkbp10, Fstl1, and Has2, which modified fibrogenesis based on their silencing, resulting in reduced Col1a1 mRNA levels and collagen accumulation in the liver. These genes fell into different groups based on the effects of their silencing on a transcriptional mini-array and histological outcomes. Silencing of Egr2 had the broadest effects in vivo and also reduced fibrogenic gene expression in a human fibroblast cell line. Prior to our study, Egr2, Atpl1a2, and Fkbp10 had not been functionally validated in fibrosis in vivo. Thus, our results provide a major advance over the existing knowledge of fibrogenic pathways. Our study is the first example of a targeted siRNA assay to identify novel fibrosis modifiers in vivo.

INTRODUCTION
Pathological tissue fibrosis is the abnormal accumulation of collagen-rich extracellular matrix (ECM) after a chronic or misregulated response to injury that progressively disrupts tissue architecture, leading to tissue stiffness, impaired organ function, and eventually organ failure. Fibrosis is featured in diverse conditions, accounts for as much as 45% of all deaths worldwide, and appears to be increasing in prevalence.1 Fibrosis complications arise in very different disease settings, from autoimmunity and environmentally induced inflammation to cancer, spanning multiple organs. To date, the treatment options are extremely limited for attenuating or reversing this process. Thus, there is an urgent need to delineate underlying pathological mechanisms that may lead to new therapeutic approaches.

Both the initiation and persistence of pathological fibrosis involves the activation and differentiation of progenitors to myofibroblasts, the key effectors in fibrosis.2 Myofibroblasts play an important role in executing physiologic tissue repair, leading to matrix deposition, wound contraction, and healing on one hand, and pathological fibrogenesis leading to chronic fibrosing conditions on the other.3 Accordingly, prominent molecular pathways in acute tissue repair often recapitulate their fibrogenic function in chronic fibrotic conditions, essentially conditions in which wound healing has gone awry; these include platelet derived growth factor receptor beta (PDGFRβ) and transforming growth factor beta (TGF-β). However, there are still many gaps in our understanding of the mechanisms underlying fibrogenesis. Due to their morphology and function, these cells are incredibly difficult to study in vitro. Recently, elegant fate mapping studies pointed to a role for pericytes as a significant progenitor pool for myofibroblasts after injury in a variety of organ systems.4 Liver pericytes, also known as hepatic stellate cells (HSCs), have been shown to be the major progenitor pool for myofibroblasts after carbon tetrachloride (CCL4)-induced liver injury and fibrosis,5,6 and gene expression profiling of these cells isolated at various time points after CCL4 administration identified molecular alterations that might be functionally relevant to fibrogenesis.6 Likewise, myofibroblasts and their precursors have been transcriptionally profiled during kidney fibrosis in mice using translational ribosome affinity purification technology.7 Isolation of this cell type induces a lot of alteration in what these cells express; therefore, it is important to study the function of the genes in their native disease environment without disrupting cell-cell and cell-matrix interactions.

We aimed to identify novel modifiers of tissue fibrosis expressed in myofibroblasts or their progenitors through RNA silencing in vivo.
Although much more challenging than conducting a cell-based screen in vitro, this in vivo approach was pursued to enable the interrogation of gene function in the context of the complex tissue environment and thereby yield physiologically relevant fibrosis modifiers. In order to achieve this objective, we employed recently generated transcriptomes from myofibroblasts and their precursors in models of liver and kidney injury and fibrosis.\textsuperscript{5,7} To achieve effective gene silencing in vivo, we used small interfering RNA (siRNA) delivery with lipid nanoparticles (siRNA-LNPs), an emerging technology for gene silencing in vivo, particularly in the liver,\textsuperscript{6} and hence deployed this technology in a model of liver fibrogenesis. The siRNA-LNP technology has been previously used to show that silencing of the collagen type I alpha 1 gene (\textit{Col1a1}) reduced its mRNA levels and collagen accumulation in liver fibrosis models.\textsuperscript{9,10} However, the use of siRNA-LNP technology in a targeted assay for identifying novel fibrosis modifiers has not yet been reported. Herein, we report our use of a platform composed of novel siRNA targets, siRNA-LNP delivery technology, and CCl\textsubscript{4}-induced liver fibrosis, resulting in the identification of novel modifiers of fibrogenesis in vivo.

**RESULTS**

**Targeted Genomic Screening Strategy**

We aimed to silence genes in key fibrogenic cell lineages, myofibroblasts, and pericytes to identify novel mediators of tissue fibrosis. Candidate genes were selected by the intersection of gene expression datasets for this cell lineage in two different organ systems. We identified genes that were commonly at least 2-fold upregulated in activated HSCs isolated from livers of mice treated with CCl\textsubscript{4} for 2 months\textsuperscript{5} and myofibroblasts and pericytes, their precursors, in the mouse kidney 2 and 5 days after unilateral ureteral obstruction (UOO).\textsuperscript{7} 169 genes met this criterion. We excluded transcripts that were not associated with a gene product, had no human homolog, or had well-known functions in fibrosis (Figure 1). Ultimately, we un-biasedly tested 24 genes by siRNA-mediated gene knockdown (KD) in vivo (Table S1).

**Establishment of CCl\textsubscript{4}-Induced Liver Fibrosis and Validation of the siRNA KD Approach in Mice**

CCl\textsubscript{4}-induced liver injury and fibrosis in mice is a well-characterized model consisting of repeated CCl\textsubscript{4} administration that injures hepatocytes, followed by a fibrogenic reaction. HSCs are activated to expand, differentiate to myofibroblasts, and produce increased levels of ECM and pro-inflammatory mediators, thereby also promoting macrophage accumulation; all of these reactions constitute a coordinated response to tissue injury and the progressive accumulation of collagen. We found that animals dosed orally with 1 mL/kg of CCl\textsubscript{4} in mineral oil on day 0 and day 7 and euthanized on day 10 exhibited significant liver fibrosis based on increased picrosirius red (PSR) and collagen immunopositivity in liver tissue as compared to vehicle-treated mice. Percent area of tissue immunoreactive with aSMA, the hallmark of myofibroblasts, and IBA-1, a macrophage-specific marker, were also increased in CCl\textsubscript{4}-treated animals, suggesting an increase in myofibroblasts and macrophage numbers in the tissue (Figures S1A–S1C). Corresponding to increased collagen deposition in tissue, \textit{Col1a1} mRNA was markedly upregulated (Figure S2A). The assessment of \textit{Col1a1} mRNA levels was used as an expedient approach to functionally assess a relatively large number of siRNA targets and flag fibrogenic genes of interest.

Given the siRNA-LNP delivery system targets multiple liver cell types, including HSCs, hepatocytes, Kupffer cells, but not endothelial cells,\textsuperscript{11–14} we validated the ability to KD genes expressed in HSCs in mice using siRNA-LNPs (Figures S1D–S1H). Mice were injected with a single dose of siRNA-LNP (1 mg/kg) against \textit{Reln}-LNPs, a gene prominently expressed in HSCs.\textsuperscript{15} Animals receiving \textit{Reln}-LNPs, but not \textit{Luc}-LNPs (negative control), showed significant reduction in \textit{Reln} mRNA in both oil- and CCl\textsubscript{4}-treated animals (Figure S1E). We also demonstrate the ability to KD the HSC-specific gene \textit{Colla1} in liver. \textit{Colla1}-LNP reduced \textit{Colla1} mRNA by 50%, with no effect on \textit{Reln} mRNA. Because the TGF-\(\beta\) pathway is a recognized fibrogenic mediator, we tested whether KD of transforming growth factor beta receptor 1 (\textit{Tgfb1}) decreased the transcription and accumulation of collagen. Indeed, administration of \textit{Tgfb1}-LNPs and \textit{Colla1}-LNPs, but not \textit{Luc}-LNPs, reduced the transcription of \textit{Colla1} mRNA (Figure S1F) as well as collagen accumulation (Figures S1G and S1H).

To test the role of genes identified in our transcriptomic analysis, we modified the siRNA administration protocol to allow pre-existing
We tested the effect of KD of 24 individual genes in vivo (Figure 2A). In the siRNA-LNP KD platform, we proceeded to test our candidate genes. Having established the suitability of the CCl4 treatment with the primary parameter for defining target genes. We similarly evaluated differences in the mRNA levels for a panel of other genes (Figure S2A). The strictly standardized mean difference (SSMD) values for Col1a1, collagen type III alpha 1 (Col3a1), tissue inhibitor of metalloproteinases 1 (Timp1), and platelet derived growth factor receptor beta (Pdgfrb), but not Tgfr1, integrin subunit alpha M (Itgam), or alpha-actin-2 (Acta2), suggested that these genes were suitable to use as readout genes (Figure S2B).

Identification of Fibrosis Modifiers

Having established the suitability of the CCl4 treatment with the siRNA-LNP KD platform, we proceeded to test our candidate genes. We tested the effect of KD of 24 individual genes in vivo (Figure 2A). We identified seven genes that significantly reduced the amount of Col1a1 mRNA (Table 1). Five of these seven genes, namely, early growth response 2 (Egr2), FK506-binding protein (Fkbp10), Na+/K+ transporting subunit alpha 2 (Atp1a2), follistatin like 1 (Fstl1), and hyaluronan synthase 2 (Has2), were silenced by 75%–100% in whole liver tissue and significantly reduced Col1a1 mRNA levels. Silencing of these genes did not elicit any overt toxicity, as measured by body weight loss (Figure S3). Because the other 19 genes did not meet these criteria (Tables 1 and S1), we focused on further analyzing the five modifiers of fibrosis.

The KD of the transcription factor (TF) Egr2 had the broadest effect by reducing the levels of Col1a1 and Col3a1 mRNA as well as the percent area immunopositive for aSMA, IBA-1, and collagen proteins, although the reduction in collagen by half did not achieve significance (Figure 2; Table 1). Egr2 silencing also reduced Pdgfrb mRNA levels (Figure 3A), suggesting a mechanism of decreased fibrosis and macrophage accumulation due to reduced expansion of activated HSCs. Given that Egr2 has structural similarities to its family members, Egr1 and Egr3, we confirmed the specificity of the Egr2 siRNA by transfecting human 293T cells with plasmids encoding mouse Egr1, Egr2, or Egr3 under the control of the CMV promoter. In the tested construct, the expression of mouse Egr1, Egr2, and Egr3 is not under the control of the endogenous promoter. Thus, reduction in gene expression is due to the binding of the siRNA Egr2 to the mRNA. Using the same Egr2 siRNA that was used for the in vivo experiments, we found that this siRNA reduced Egr2 mRNA levels, but not Egr1 or Egr3 mRNA, unambiguously demonstrating that Egr1 and Egr3 were not targeted by our Egr2 siRNA (Figures S4A–S4C). Interestingly, even though the siRNA against Egr2 was specific, Egr2-LNP treatment in vivo also reduced Egr1 and Egr3 mRNA levels (Figures S4D–S4F), suggesting transcriptional co-regulation of EGR family members.

Similar to Egr2, KD of Fkbp10 or Atp1a2 reduced the collagen and IBA-1 positive areas as did that of Egr2 (Figure 2; Table 1); however,
KD of Fkbp10 or Atp1a2 did not alter the percent immunopositivity for aSMA. Fkbp10 KD also decreased Pdgfrb mRNA levels, but this effect was somewhat weaker than that of Egr2 (28% versus 45% reduction; Figures 3A and 3B), possibly accounting for its lack of effect on aSMA immunopositivity. Atp1a2 KD also decreased Timp1 mRNA levels (Figure 3C). These results suggest that Egr2, Fkbp10, and Atp1a2 regulate fibrogenesis through different mechanisms.

KD of either of the two other fibrosis modifiers, Fstl1 and Has2, decreased Col1a1 mRNA levels as well as macrophage and collagen accumulation in the tissue, although Has2 KD did not achieve significance for the latter (Figure 2). Notably, KD of these genes showed increased amounts of aSMA immunopositivity, which was highly significant for Has2 KD, suggesting increased numbers of myofibroblasts. Corresponding with the increased myofibroblasts, we detected higher levels of the Timp1 mRNA in the livers of both Fstl1 and Has2 KD animals (Figures 3D and 3E). Despite the increased myofibroblast and Timp1 mRNA levels, Fstl1 KD significantly reduced collagen accumulation. In order to understand these findings, we also conducted in parallel in vitro studies and showed that three independent FSTL1 siRNAs reduced COL1A1 mRNA levels. Thus, the direct reduction of collagen transcription may be sufficient to reduce overall collagen accumulation, despite the elevated number of myofibroblasts and reduced collagen degradation (Figure S5).

Silencing of EGR2 in Human HSCs Reduces Expression of Fibrogenic Genes

EGR2 emerged as the strongest modifier of fibrosis in the CCl4 liver fibrosis/siRNA KD platform. We aimed to directly demonstrate that EGR2 KD in an HSC would reduce COL1A1 and PDGFRB mRNA levels. We tested the effect of EGR2 KD in the human HSC line LX-2 using three independent siRNAs (Figure 4). This experiment required a different siRNA to be used because no species cross-reactive siRNA was designed (see Materials and Methods). All siRNAs against ERG2 reduced its mRNA levels as well as COL1A1 and PDGFRB mRNA levels. These results are consistent with the effect of Egr2 KD in our in vivo CCl4 assay platform. As expected, KD of COL1A1 reduced its own expression, but not that of EGR2. Interestingly, COL1A1 silencing also reduced PDGFRB mRNA levels, suggesting a regulatory feedback loop. Taken together, these data support that EGR2 is fibrogenic in both rodents and humans.

DISCUSSION

There are currently limitations in the understanding of pathophysiological mechanisms underlying fibrotic diseases. Myofibroblasts are key effectors of fibrosis and therefore the elucidation of pathways that promote this cell lineage would be a significant advance in the field. Here, we report the use of a targeted in vivo siRNA KD platform to identify novel mediators of fibrogenesis. We leveraged novel biology-targeting genes upregulated during liver and kidney fibrosis in myofibroblasts and their progenitors and siRNA-LNP technology to silence these genes in CCl4-induced liver injury and fibrosis in mice. We identified five genes, namely Egr2, Atp1a2, Fkbp10, Fstl1, and Has2, which modified fibrogenesis in this system because their KD reduced Col1a1 mRNA levels and collagen accumulation in the liver. Prior to our study, Egr2, Atp1a2, and Fkbp10 had not been functionally validated in fibrosis in vivo, and our study is the first to validate Fstl1 and Has2 in liver fibrosis. We further directly demonstrated that Egr2 KD in human HSCs reduced the expression of fibrogenic genes. We conclude that in vivo siRNA KD using siRNA-LNP is a powerful tool to identify disease-relevant modifiers in vivo.
circulating fibrocytes in situ. Myofibroblasts respond to chemomechanical stimuli, and therefore disruption of their native environment during cell isolations alters their activation state. To circumvent these shortcomings, we silenced target genes in vivo by systemic administering siRNA-LNPs. This approach allowed us to study functional consequences of silencing of specific genes of interest in vivo in their native environment. It is of note that our siRNA-LNP delivery approach allows us to silence gene expression in HSCs; however, this LNP formulation also targets other cells in the liver, including Kupffer cells and hepatocytes, but not endothelial cells. Therefore, we cannot rule out the contribution of gene silencing in Kupffer cells and hepatocytes on the anti-fibrotic outcome.

Our strategy to identify novel players in tissue fibrosis took advantage of recent transcriptomic datasets for myofibroblasts and their precursors isolated from fibrotic liver or kidney in mouse models. Using siRNA-LNP delivery technology, we tested the effect of gene silencing in a physiologically relevant context in vivo. We employed a CCl₄ administration model that featured significant collagen accumulation and established several robust RNA and histological measures of fibrogenic activity to identify fibrosis modifiers. Using the criteria of significant reduction of Col1a1 mRNA levels, we identified seven modifiers of interest out of 24 tested genes. Of greatest interest are those that also reduced collagen accumulation in the tissue, namely, Egr2, Atp1a2, Fkbp10, Fstl1, and Has2. Notably, these five genes were the most strongly silenced in vivo (by 75% or greater) that significantly reduced Col1a1 mRNA levels. It is possible that we might have achieved a greater degree of in vivo gene silencing for other target genes with a higher dose or a different dosing paradigm. Thus, although we cannot draw conclusions about the functional role of genes whose silencing was incomplete, our results clearly identify five genes as novel fibrogenic mediators in liver fibrosis in vivo. These

Figure 3. Effect of Gene Silencing on mRNA Levels of a Panel of Readout Genes

(A–E) qPCR data of indicated readout genes (x axis) of animals that have received siRNA-LNPs targeting Egr2 (A), Fkbp10 (B), Atp1a2 (C), Fstl1 (D), or Has2 (E) were plotted as percentage of Luc-LNP treated animals within the same experiment. n = 4–15 animals per group. Data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA and Dunnett’s post test. Statistical differences are indicated as *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001. ND, not detected.
Egr2, a TF of the EGR family, emerged as the strongest effector in the siRNA screen. EGR TFs are important for the induction of cellular programs, including cell proliferation, differentiation, and death, in response to stimuli such as cytokines, growth factors, and toxic substances.\(^{17–19}\) Egr2 has not been previously reported to play a role in tissue fibrosis in vivo.\(^ {20}\) Rather, EGR2 is prominently known for its role in early myelination of the peripheral nervous system in humans and mice.\(^ {21–23}\)

Egr2 KD silenced its expression by 94%, reduced Col1a1 and Col3a1 mRNA levels, and diminished accumulation of myofibroblasts and macrophages, each to 15% of control. Liver fibrosis was also alleviated because collagen accumulation was reduced by half. Thus, our demonstration of reduced fibrogenic activity by Egr2 silencing reveals a novel EGR2 function. The downregulation of Pdgfb mRNA levels by 45% suggests that EGR2 may promote HSC expansion. This mechanism would explain the reduced aSMA-expressing HSC and thereby reduced signals for macrophage accumulation, all of which may have contributed to the reduction in fibrosis.

Importantly, we also directly demonstrate that EGR2 KD in a human HSC line reduces COL1A1 and PDGFRB transcripts, supporting the regulation of a fibrogenic program in HSCs by this TF. The comparatively weaker effect of COL1A1 silencing in vitro (~50%) versus in vivo siRNA-LNP-mediated KD (80%) of EGR2 is due to the use of different delivery methods as well as the use of different siRNA sequences to achieve gene silencing in human cells versus in vivo in mice. Interestingly, COL1A1 KD also reduced PDGFRB mRNA levels. Because ECM accumulation and expression of PDGFRB are linked,\(^ {24}\) it is feasible that decreased collagen production would decrease PDGFRB expression in a feedback loop. The relevance of our finding to fibrogenesis in humans is also supported by a prior in vitro study, in which overexpression of Egr2 in human skin fibroblasts upregulated collagen gene expression and myofibroblast differentiation, and these profibrotic TGF-β-induced responses were attenuated by Egr2-depletion.\(^ {25}\)

Relevant genes fell into different groups based on the effects of their silencing on a mini mRNA array and histological measures. We propose grouping them as follows: Egr2, a gene with potential function in HSC expansion; Atp1a2 and Fkbp10, genes that regulate mechano-sensing; and Fstl1 and Has2, genes involved in matrix-cell signaling.

Figure 4. KD of EGR2 Reduces COL1A1 and PDGFRB mRNA Levels in Human HSC Line

(A–C) Graphs show the effect of siRNA-KD of the indicated genes (x axis) on relative mRNA levels of EGR2 (A), COL1A1 (B), and PDGFRB (C). The effect of three independent siRNAs against EGR2 is shown. Data are presented as mean ± SD. Statistical comparisons were performed using one-way ANOVA and Dunnett’s post test. Statistical differences are indicated as ***p < 0.001 and ****p < 0.0001. ns, not significant.
Atp1a2 and Fkbp10 were identified as fibrogenic modulators, with mechanisms of action distinct from that of Egr2. ATP1A2 is the large catalytic subunit of an enzyme that catalyzes the hydrolysis of ATP, coupled with the exchange of Na⁺ and K⁺ ions across the plasma membrane. ATP1A2 has a known function in the contractility of skeletal and cardiac muscle. On the basis of our results, it is intriguing to speculate that Atp1a2 silencing may have reduced the contractility of myofibroblasts, effectively mimicking their behavior in a more compliant matrix environment and hence resulting in a less fibrogenic program.31,32 Supporting this theory, we found that Atp1a2 KD reduced fibrogenesis in vivo by significantly limiting the transcription of the Col1a1 and Timp1 genes, macrophage accumulation, and collagen deposition. However, in contrast to Egr2, Atp1a2 silencing had no effect on aSMA expression. This pattern suggests that the myofibroblasts are generated but have reduced mechanosensing function. This theme of altered mechanosensing may also explain the alleviated fibrosis upon silencing of Fkbp10. FKBP10 is a molecular chaperone in the endoplasmatic reticulum that directly interacts with collagen I, contributing to the maturation and molecular stability of type I procollagen, promoting normal secretion, stable inter-molecular crosslinking, and collagen deposition in the ECM.33 FKBP10 has a well-established role in bone mass in humans and mice, but its role in tissue fibrosis is largely unexplored.34 Fkbp10−/− mouse embryos are post-natally lethal and display reduced collagen crosslinking in calvarial bones.35 In this study, we report our novel finding that Fkbp10 silencing reduces collagen deposition by 64%; however, we found no reduction in aSMA expression. We speculate that Fkbp10 silencing alters the quantity or crosslinking of the ECM and thereby alters mechanosensing. Consistent with our in vivo results, a prior in vitro study showed that inhibition of FKBP10 in primary human fibroblasts from pulmonary fibrosis patients attenuates expression of pro-fibrotic genes and decreases collagen secretion.36 Thus, our findings expand the scope of Atp1a2 and Fkbp10 functions by demonstrating their novel roles in fibrogenesis in vivo.

Fstl1 and Has2 silencing also affected multiple fibrogenic readouts in our assay platform, but resulted in a pattern distinct from that of Egr2, Atp1a2, and Fkbp10. Both Fstl1 and Has2 silencing reduced Col1a1 mRNA, macrophage accumulation, and collagen deposition but counterintuitively increased myofibroblast density as well as the level of Timp1 mRNA, a profibrotic pathway. FSTL1 is a TGF-β-inducible, secreted, matricellular glycoprotein belonging to the secreted protein acidic rich in cysteines (SPARC) family, with pleiotropic functions.37–39 During the course of our current study, it was reported that haplodepletion of Fstl1 or a neutralizing anti-FSTL1 antibody reduced bleomycin-induced pulmonary fibrosis.38 We found that Fstl1 gene silencing reduced collagen transcription and accumulation in the context of liver fibrosis but surprisingly increased myofibroblast density, possibly reflecting a compensatory feedback loop. We hypothesize that the overall reduction in collagen accumulation is a direct result of decreased collagen production by Fstl1 silencing, so that the increased number of myofibroblasts and reduced activity of collagen degradation would still result in decreased overall collagen accumulation. Indeed, KD of FSTL1 in the human HSC line LX-2 resulted in decreased levels of COL1A1 mRNA.

Like Fstl1, silencing of Has2 resulted in unexpected effects, also likely through a matrix-modifying mechanism. HAS2 is one of three isoenzymes responsible for cellular hyaluronan (HA) synthesis, a matrix component normally highly abundant in joint synovium. HA is well known for its role in wound healing.30,41 Has2 has been previously reported to be pathological in the bleomycin-induced lung fibrosis model, in which mesenchymal cell-specific deletion of Has2 abrogated myofibroblast accumulation and inhibited the development of lung fibrosis.34 We show that Has2 silencing also reduces liver fibrosis. Has2 silencing also unexpectedly augmented myofibroblast density. Taken together, our studies have identified Fstl1 and Has2 as fibrosis modifiers in our in vivo liver fibrosis platform and reveal surprising effects of silencing these genes, supporting their complex role in the regulation of fibrogenesis.

To the best of our knowledge, our study is the first example of a targeted siRNA assay for the discovery of novel fibrosis modifers in vivo. The results implicate five novel modulators of the fibrogenic process in the liver and demonstrate the feasibility of siRNA-LNP for interrogating gene function in the liver. Because these genes are commonly upregulated in pericytes and myofibroblasts in both liver and kidney fibrosis models, these genes warrant follow-up to further explore their function and mechanism of action in liver and other organs and to inform their role in both physiological and pathophysiological fibrosis.

MATERIALS AND METHODS

Animal Experiments

All animal procedures were conducted in accordance with Cambridge laws and the Institutional Animal Care and Use Committee approved protocol No. 657. 8- to 10-week-old male BALB/c mice (Taconic Bioscience) were given CCl₄ by oral gavage (Sigma-Aldrich; 1 mL/kg dissolved in mineral oil) on days 0 and 7. Mineral oil alone was used as a control. In some experiments, siRNA-LNPs were given by tail vein injection. siRNA-LNPs were either given on day 6 at a concentration of 1 mg/kg or on days −7, −3, 2, and 6 at 0.5 mg/kg. These experiments were controlled with cohorts of mice that received siRNA targeting Luciferase (Luc-LNPs) with oil or CCl₄, respectively. Animals were sacrificed on day 10 by CO₂ asphyxiation, followed by cardiac perfusion with 10 mL of PBS, and liver lobes were harvested for qPCR and histopathological analysis.

Gene Selection

Target fibrosis modifier genes were selected by intersecting transcriptional data generated from isolated HSCs of mice treated with CCl₄ for 2 months, and genes specifically expressed in pericytes/myofibroblasts in the mouse kidney 2 and 5 days after UUO.6,7 169 genes were commonly to both datasets and significantly upregulated at least 2-fold. Among these, we excluded transcripts that were not associated with a gene product, genes with no known human homolog, and
well-known modifiers in fibrosis (validated in vivo by independent investigators), resulting in 24 genes for further evaluation.

**siRNA Design and Synthesis**

A set of ten siRNAs with canonical siRNA structures was defined for each target gene. All siRNAs were directed against the coding sequence of their respective target mRNA and perfectly matched all known mRNA transcript variants of the target gene available in the NCBI reference sequence database (RefSeq DB, release 65, May 2014).

The siRNAs used in this study were designed to be a perfect match only to their target mRNA and to have ≥ 2 mismatches within positions 2–18 of the 19-mer sequence to any other genes. The design as described above is sufficient to ensure specificity. Specifically, at least four mismatches between the siRNA sequences of each of the genes of interest (Atp1a2, Egr2, Fkbp10, Fstl1, and Has2) and the readout genes Col1a1, Col3a1, Tgfr1, Pdgfrb, and Timp1. Further, there were three mismatches between Egr2 siRNA and Egr1 sequence and more than four mismatches between Egr2 siRNA and Egr3 sequence.

The siRNA antisense strands lacked a seed region (nucleotides 2–7) identical to a seed region (nucleotides 2–7) of known mouse miRNAs (miRBase, release 20, June 2013). Additional similarity analysis showed that there were at least four mismatches between the siRNA sequences of each of the genes of interest (Atp1a2, Egr2, Fkbp10, Fstl1, and Has2) and the readout genes Col1a1, Col3a1, Tgfr1, Pdgfrb, and Timp1. Further, there were three mismatches between Egr2 siRNA and Egr1 sequence and more than four mismatches between Egr2 siRNA and Egr3 sequence. From siRNAs fulfilling those criteria, the siRNAs for each final screening were selected for predicted activity based on analysis with proprietary algorithms (Axolabs, GmbH). In vivo grade siRNAs were synthesized by Axolabs, as described elsewhere.

**siRNA In Vitro Screen**

Each of the ten aforementioned siRNA molecules/target gene was screened for its potency to KD gene expression in a cell-based reporter assay. For each target gene, the regions of their coding sequences targeted by the siRNAs were synthesized and inserted to the 3’ UTR of the firefly luciferase gene in pCMVluc(V) validation vector (Origene), generating an mRNA transcript that encodes both the luciferase (Luc) and the target gene when expressed in cells. 293T cells (ATCC) were co-transfected with the validation vector (Origene), generating an mRNA transcript that encodes both the luciferase expression and the siRNAs of interest using Lipofectamine 2000 (Thermo Fisher Scientific), and luciferase signal was detected using a Promega System (Promega), and luciferase expression was measured 24 hr post-gene (>90% luciferase reduction at 1 nM siRNA in vitro) was then formulated as LNPs for in vivo application. The sequences of the siRNAs that resulted in efficient KD (≥75%) of their target gene in vivo are provided in Table S2.

**siRNA LNP Formulation**

siRNA-LNPs were synthesized by mixing together an siRNA-containing aqueous phase with a lipid-containing ethanol phase. The aqueous phase contained siRNA in 10 mM citrate buffer (pH 3). The ethanol phase contained ionizable lipid. C12-200 (Wuxi AppTec), 1,2-distearoyl-sn-glycerol-3-phosphocholine (DSPC, Avanti Polar Lipids), cholesterol (Sigma), and 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (C14 PEG 2000, Avanti) at a 50:10:38:5:1.5 molar ratio and 5:1 C12-200:siRNA weight ratio. The aqueous and ethanol phases were mixed together at a 3:1 volume ratio in a microfluidic chip device using syringe pumps as previously described to a final siRNA concentration of 1 mg/mL. The resultant siRNA-LNPs were dialyzed overnight in a 20,000 molecular weight cut-off (MWCO) cassette against 1x PBS at 4°C. On average, siRNA-LNPs had a mean diameter of approximately 100–150 nm, with a polydispersity index between 0.1 and 0.2 as measured by Dynamic Light Scattering (ZetaSizer, Malvern Instruments). The siRNA encapsulation efficiency (approximately 60%) was determined using a modified Quant-iT Ribogreen Assay (Invitrogen) as previously described. This siRNA delivery system can target multiple liver cell types, including hepatocytes, monocytes, tissue macrophages, dendritic cells (DCs), and myofibroblasts/pericytes.

**RNA Expression Analysis**

RNA was extracted from liver lobes with Trizol (Thermo Fisher Scientific), followed by on-column DNase digestion using RNasey Mini Kits and RNase free DNase sets (both from QIAGEN). cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit, and qPCR analysis was performed using pre-designed TaqMan primer probes (Table S3) on the QuantStudio 12K Flex Real-Time PCR System (all from Thermo Fisher Scientific) to determine the expression levels of siRNA target genes and a panel of readout genes. All procedures were conducted following the manufacturer’s protocols. The relative expression of each gene was normalized to mouse 18S rRNA by the 2−ΔΔCT method, and the mean expression of each gene in the control group was set to 100%.

**Histopathology**

Livers lobes were fixed in 10% neutral buffered formalin and embedded in paraffin. 5-μm sections were stained with PSR using an automated Leica system according to standard protocols. Immunohistochemistry (IHC) was performed with the Ventana system. Anti-alpha-Smooth muscle actin (aSMA) IHC was performed with fluorescein isothiocyanate (FITC)-labeled mouse monoclonal antibody 1A4, followed by an anti-FITC antibody and periodic acid-Schiff (PAS) counterstain (Abcam). Anti-IBA-1 and anti-COL1A1 IHC was performed using rabbit polyclonal antibodies (cat. # 019-19741, Wako, and Genetex, cat. # GTX41286).
Image analysis was performed by writing custom algorithms with Visiopharm software. The IBA-1 positive area was limited to infiltrating macrophages by differentiating isolated single cells (largely Kupffer cells lining sinusoids) from larger round cells in clusters (peribortal, largely infiltrating macrophages, sometimes filled with mineral). De novo interstitial aSMA was differentiated from endogenous aSMA in muscular arteries by performing a PAS counterstain and then programming the algorithm to distinguish vascular morphology for exclusion from quantification. Exclusion of muscular arteries was similarly performed on sections with anti-COL1A1 IHC.

siRNA Gene Silencing in Cell Lines

The human HSC line LX-2 (EMD Millipore) was transfected with siRNAs against EGR2, COL1A1, or Luc with Lipofectamine RNAiMAX reagent following the manufacturer’s protocol. We used the following predesigned siRNAs (listed as siRNA ID): EGR2: s4540, s4541, and s4542; COL1A1: s3276; Luc: AM4629; and FSTL1: s22032, s22033, and s22034 (Thermo Fisher Scientific). RNA was isolated on day 2 after transfection using RNaseasy kits with on-column DNase digestion (QIAGEN). cDNA synthesis and qPCR was performed as stated above.

293T cells (ATCC) were co-transfected with pCMV-Egr1, pCMV-Egr2, or pCMV-Egr3 (Origene) and either pCMV-Egr1, pCMV-Egr2, or pCMV-Egr3 (Origene) and either Silencer Select Negative Control No. 1 siRNA (Thermo Fisher Scientific) or siRNA against Egr2 (Axolabs). RNA was isolated 24 hr after co-transfection using RNaseasy kits plus kits (QIAGEN). cDNA synthesis and qPCR was performed as stated above.

Statistical Analysis

Datasets are presented as mean ± SD. Statistical significance was assessed by two-tailed, unpaired Student’s t test for comparison of two groups or by one-way ANOVA, followed by the Dunnett’s multiple comparisons test for more than two groups. Differences with adjusted P values of less than 0.05 were considered statistically significant. Non-adjusted P values were listed to convey trends (Table 1).

We used an SSMD method to analyze the suitability of the mRNA readout genes in our siRNA-LNP/CCl4 liver fibrosis platform (Figure S2). The SSMD method was used to calculate the median of differences divided by the standard deviation of the differences between the group treated with oil and the control siRNA targeting Luciferase (oll/Luc-LNP) and versus CCL4/Luc-LNP. It represents the average fold change penalized by the variability of the fold change across animals. SSMD is a suitable method because it takes the variability between animals into account. For our study, a threshold of β < 1.5 was taken as the cut-off value for suitable readout genes.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.04.014.

AUTHOR CONTRIBUTIONS

E.H.V., D.G.A., V.K., J.D., and L.C.B. designed the study; E.H.V., L.C.B., A.A., T.R., and I.D.-L. conducted experiments; M.H., K.J.K., and F.F.M. generated critical reagents; E.H.V., L.C.B., and S.H. analyzed data; T.O.C., T.N., T.K., and D.B. reviewed and edited the manuscript; E.H.V. and L.C.B. wrote the manuscript.

CONFLICTS OF INTEREST

E.H.V., T.R., S.H., I.D.-L., T.O.C., and L.C.B. are employees and stockholders of Biogen. The remaining authors declare no conflicts of interest.

ACKNOWLEDGMENTS

We want to thank Wenting Wang (Biogen, Inc.) for statistical advice, Danny Sofia (Biogen, Inc.) for image analysis support, and J. Robert Dorkin (MIT) for formulating siRNAs as LNPs. D.B. was supported by the following grants: AA011999, ES010337, and GM041804.

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