Development of novel nucleic acid therapy aimed at directly controlling liver fibrosis

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Currently, no drugs directly treat liver fibrosis. Previously, we have shown that treatment with miR-29a-3p improved liver fibrosis in a mouse model. To investigate the effectiveness of nucleic acid therapy at a lower dose, a modified nucleic acid was prepared based on miR-29a-3p. The original microRNA was changed to an RNA-DNA hybrid structure: the $2'$ position of the RNA was modified with a fluorine base, and locked nucleic acid and phosphorothioate were crosslinked (hereafter called modified nucleic acid). In a mouse model of chronic liver disease treated with carbon tetrachloride (CCl_4), the inhibitory effect on liver fibrosis was evaluated with oral administration of the modified nucleic acid. The modified nucleic acid was detected in the liver and gastrointestinal tract within 15 min of oral administration. After 5 weeks of stimulation with CCl_4, oral administration of the modified nucleic acid for 2 weeks improved liver fibrosis; CCl_4 stimulation was continued during this period as well. This treatment also suppressed the worsening of liver fibrosis. We developed a method to improve liver fibrosis orally using nuclease-resistant nucleic acids without using a drug delivery system. This method may be used as a new treatment for inhibiting the progression of liver fibrosis.

INTRODUCTION

Repeated cycles of liver cell damage, proliferation, and inflammation result in liver fibrosis due to the overproduction of extracellular matrix (ECM) components, such as type 1 collagen (COL1), by activated hepatic stellate cells (HSCs), eventually leading to liver cirrhosis.[1–3] COL1 is the most abundant component of the ECM and is produced by the COL1A1 and COL1A2 genes.[4] Furthermore, >90% of hepatocellular carcinomas (HCCs) develop in the presence of fibrosis or cirrhosis, and improving liver fibrosis is extremely important not only for maintaining liver function but also for preventing liver carcinogenesis.[5]

From the perspective of fibrosis progression and carcinogenesis, several studies have focused on the abnormal expression of platelet-derived growth factor C (PDGFC) in the liver. A transgenic mouse model, in which PDGFC was ectopically expressed in hepatocytes, developed progressive liver fibrosis with a high incidence of HCC. PDGFC alters the liver microenvironment and causes epithelial tumors.[6–8] PDGF also promotes fibrosis not only in the liver but also in many organ systems, such as the lungs, vascular system, skin, and liver.[9] In a normal human liver, the expression of platelet-derived growth factor A (PDGFA), platelet-derived growth factor B (PDGFB), and PDGFC is relatively low, but it is frequently elevated in cirrhotic livers and in HCC.[10,11] Administration of miR-214, which targets PDGFC, improves liver fibrosis.[12] Supplementation with miR-29a in a mouse model of chronic liver disease regulates the expression of COL1A1 and PDGFC, regulates HSC activation, and improves fibrosis in damaged liver tissue.[13] Overexpression of miR-29a-3p targets fibrinogen like 2 (FGL2), mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4), interleukin 1B (IL-1β), N-myc downstream regulated 1 (NDRG1), and BCL2 apoptosis regulator (BCL2), in addition to COL1A1 and PDGFC, and exerts anti-inflammatory effects, enhances apoptosis, and inhibits angiogenesis.[13]

MicroRNAs are small endogenous noncoding RNAs that regulate gene expression by degrading or suppressing the translation of target mRNAs.[14] This has been shown to be associated with abnormal microRNA expression in many diseases. MicroRNAs are involved in numerous physiological and pathophysiological processes, such as cancer,[15,16] cell cycle progression,[17] infectious diseases,[18] diabetes,[19] metabolism,[20] and fibrosis.[21]

Drug discovery using microRNAs, based on nucleic acid stabilization, is also underway. The main problems with the development of oligonucleotide therapeutics are difficulty in efficient delivery to target organs and tissues, except the liver, and difficulty in achieving off-target interactions.[22–24] The liver is a highly perfused tissue, and the presence of a discontinuous sinusoidal endothelium can lead to the uptake

A

| miR-29a-3p | 3' auuggcuaaAGUCUACCACGAu 5' |

B

miR-29a-3p	3' auuggcuaaAGUCUACCACGAu 5'
	‖‖ ‖‖‖‖
COL1A1	910:5'gggagggaaUCA-CUGGUGCUa 3'

miR-29a-3p	3' auuggcUAA-AGUCUACCACGAu 5'
	‖‖ ‖‖: ‖‖‖‖‖
PDGFC	922:5'uauuucAUUCUCGUAUGGUGCUa 3'

F

GC1	T(L)^A(L)^G(L)^c^a^c^c^a^g^g^c^t^g^T(L)^C(L)^C(L)
GC2	T(L)^A(L)^G(L)^c^a^c^c^a^t^c^g^t^t^G(L)^C(L)^C(L)
GC3	T(L)^A(L)^G(L)^c^a^c^c^a^g^c^a^t^c^A(L)^C(L)^C(L)
GC4	C(L)^C(L)^C(L)^t^t^a^g^c^a^c^c^a^t^C(L)^G(L)^T(L)

G

GP1	T(L)^A(L)^G(L)^c^a^c^c^a^g^c^g^c^c^C(L)^T(L)^A(L)
GP2	T(L)^A(L)^G(L)^c^a^c^c^a^t^a^c^g^a^G(L)^A(L)^A(L)
GP3	T(L)^A(L)^G(L)^c^a^c^c^a^c^a^t^a^a^C(L)^C(L)^T(L)
GP4	A(L)^C(L)^T(L)^c^t^a^g^c^a^c^c^a^t^A(L)^C(L)^G(L)

E

seed 1-1	T(L)^a^g^C(L)^a^c^C(L)
seed 1-2	T(L)^a^G(L)^c^A(L)^c^C(L)
seed 2-1	A(L)^g^c^A(L)^c^c^A(L)
seed 2-2	A(L)^g^C(L)^a^C(L)^c^A(L)

C

siC-1	AGUGGUUUGGAUGGUGCUAtt
	UAGCACCAUCCAAACCACUtt
siC-2	GAUGGACAGCCUGGUGCUAtt
	UAGCACCAGGCUGUCCAUCtt
siC-3	CCCGGCAACGAUGGUGCUAtt
	UAGCACCAUCGUUGCCGGGtt
siC-4	CCUGGUGAUGCUGGUGCUAtt
	UAGCACCAGCAUCACCAGGtt
siC-5	ACUGGUUUCCCUGGUGCUAtt
	UAGCACCAGGGAAACCAGUtt

D

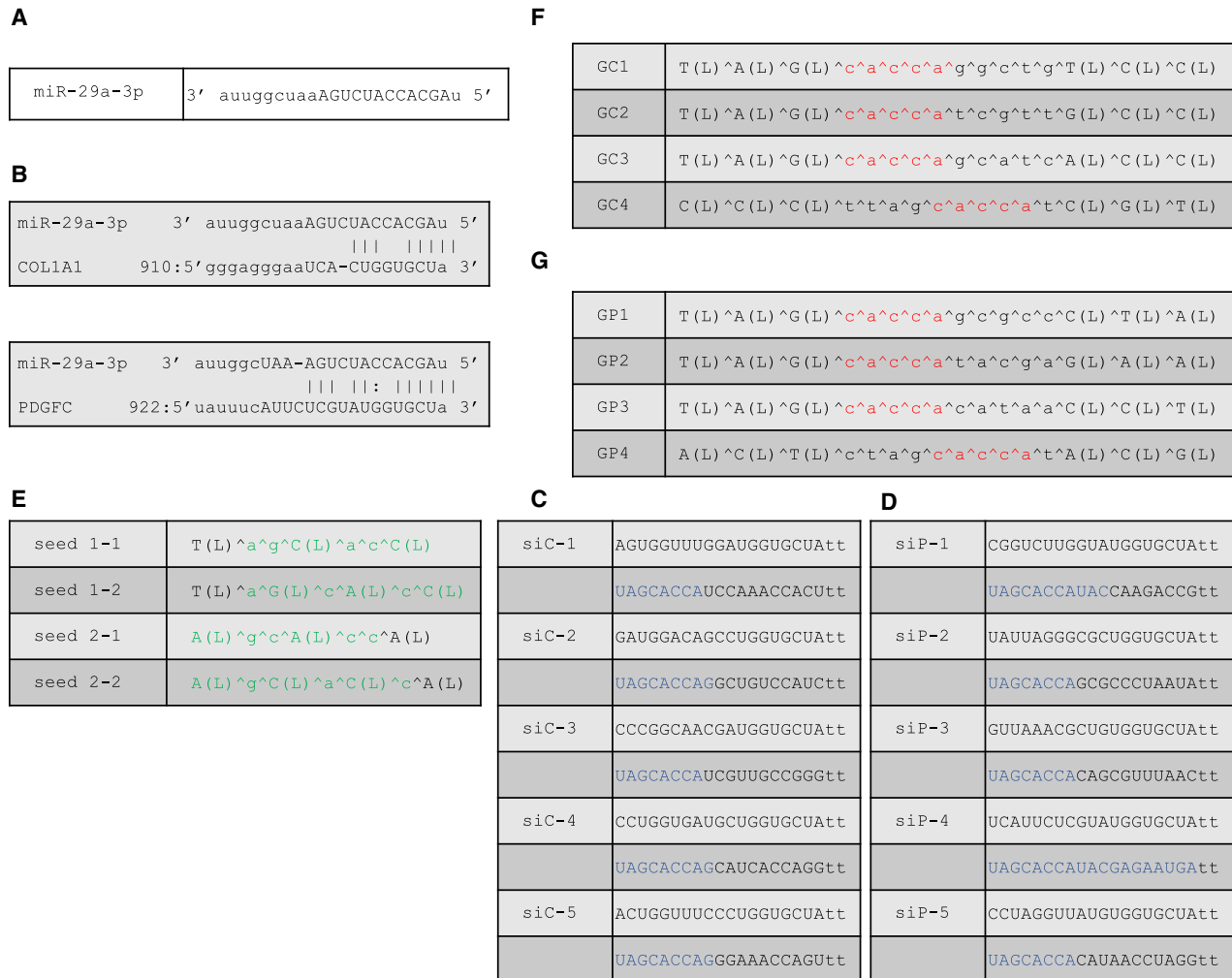| siP-1	CGGUCUUGGUAUGGUGCUAtt
	UAGCACCAUACCAAGACCGtt
siP-2	UAUUAGGGCGCUGGUGCUAtt
	UAGCACCAGCGCCCUAAUAtt
siP-3	GUUAAACGCUGUGGUGCUAtt
	UAGCACCACAGCGUUUAACtt
siP-4	UCAUUCUCGUAUGGUGCUAtt
	UAGCACCAUACGAGAAUGAtt
siP-5	CCUAGGUUAUGUGGUGCUAtt
	UAGCACCACAUAACCUAGGtt

Figure 1. Construction of a modified nucleic acid of hsa-miR-29a-3p

(A) hsa-miR-29a-3p is the original nucleic acid sequence (capitalized letters indicate the seed sequence). (B) Relationship between the microRNA sequence and target gene recognition site. Shown at the top and bottom are the COL1A1 and PDGFC target recognition sequences, respectively. Blue letters indicate sequences that recognized the target genes. Uppercase letters indicate complementary base sequences of microRNAs and target genes in (A) and (B). (C and D) Modified siRNA sequence of (C) COL1A1 and (D) PDGFC. The sequence of each double strand is shown. (E) Nucleic acid structure based on the microRNA seed sequence. The uppercase and lowercase letters indicate RNA and DNA, respectively. N(L) shows LNA modification, green letters indicate the seed sequence, and ^ indicates thiophosphate crosslinking. (F) RNA-DNA hybrid structure for COL1A1 recognition. The uppercase and lowercase letters indicate RNA and DNA, respectively. N(L) shows LNA modification, red letters indicate the seed sequence, and ^ indicates thiophosphate crosslinking. (G) RNA-DNA hybrid structure for PDGFC recognition. Array notations from: N, RNA; n, DNA; N(L), LNA; COL1A1, collagen, type I, alpha 1; LNA, locked nucleic acid; PDGFC, platelet-derived growth factor C.

of free oligonucleotides before renal excretion.[25] The liver also contains very high concentrations of receptors, such as scavenger and asialoglycoprotein receptors, that may promote uptake or rapid reuse.[26,27] Therefore, if degradation of nucleic acids by ribonuclease is avoided, then it will not be difficult for nucleic acids to reach the liver after administration.

We have shown previously that regulating miR-29a, which simultaneously regulates multiple targets in the liver in chronic liver diseases, is a useful therapeutic method. In this study, we modified

and stabilized the structure of miR-29a and attempted to develop a nucleic acid that can reach the liver without a drug delivery system (DDS).

RESULTS

Nucleic acid optimization to maximize the target suppression effect

Nucleic acid structure optimization for stability was performed based on the seed sequence of miR-29a-3p (Figure 1A) and sequences targeting COL1A1 and PDGFC (Figure 1B). The small interfering

RNA (siRNA) structure targeting the COL1A1 sequence (siC-1–siC-5; Figure 1C) was chosen to perfectly match the target site of COL1A1. The siRNA structure targeting the PDGFC sequence (siP-1–siP-5; Figure 1D) was chosen to perfectly match the target site of PDGFC. A nucleic acid sequence containing a seed sequence of miR-29a-3p was created. Both ends of the nucleic acid were modified with locked nucleic acid (LNA) to create an RNA-DNA hybrid (seed 1-1-2-2; Figure 1E). RNA-DNA hybrid structures targeting COL1A1 (GC1–GC4; Figure 1F) and PDGFC (GP1–GP4; Figure 1G) were prepared. In RNA-DNA hybrid nucleic acids, each nucleic acid is linked to a phosphorothioate. The DNA structure is the sequence of miR-29a-3p, and we selected a structure that included a site (CACCA) that binds to the target gene. Nucleic acids with four sequences were generated for COL1A1 and PDGFC.

In cells collected after 24 h, the siRNA targeting COL1A1 had a suppressive effect on the target gene regardless of transforming growth factor β (TGF-β) treatment. Among siRNAs targeting PDGFC, siP-5 had the greatest effect of suppressing target genes. In cells collected after 48 h, the siRNA targeting COL1A1 had an overall suppressive effect on the target gene. Among siRNAs targeting PDGFC, siP-5 suppressed the target gene. The RNA-DNA hybrid nucleic acid targeted COL1A1 and suppressed COL1A1 expression for 24 and 48 h; however, diffusion-targeting PDGFC had a poor suppressive effect on PDGFC expression. The seed nucleic acid had a target gene suppression effect after both 24 and 48 h in the absence of TGF-β treatment, but the target gene suppression effect was poor with TGF-β treatment (Figures S1 and S2).

Anti-fibrosis effect with the modified nucleic acid *in vivo*

Based on the results of *in vitro* experiments, the effect of nucleic acids with siRNA and RNA-DNA structures was evaluated *in vivo*. We decided to proceed with analysis of siP-5 as an siRNA and GC1 as a hybrid structure. A model of chronic liver disease was prepared by intraperitoneally administering CCl_4 to mice for 5 weeks; 10 μg nucleic acid mixed with atelocollagen was administered via the tail vein twice a week after completion of CCl_4 administration (Figure S3A).

The expression of Col1a1 in the liver was suppressed by miR-29a-3p and GC1 nucleic acids compared with the negative control (Figure S3C). Further, the picrosirius red (PSR)-positive area decreased significantly with miR-29a-3p but not with GC1 or siP-5 (Figures S3B–S3D).

Target gene inhibitory effect with nucleic acid modification

We optimized the nucleic acid to achieve both the target gene suppression effect with GC1 and stability of the nucleic acid. Twelve modified nucleic acids were prepared as follows: 3 types of 2′-fluoro (F) modification; 2, 2′-O-methylation2′-OMe); 2, 2′-O-methoxyethyl (2′-MOE); 2, 2′-F and OMe; 2, 2′-F and MOE; and 1, 2′-F, OMe, and MOE (Figure S4A). We compared the expression of COL1A1 and PDGFC using LX2 cells with and without TGF-β stimulation for each gene. Regarding COL1A1, miR-29a-3p and GC1_F_#2 suppressed the target gene both with and without TGF-

β stimulation. In contrast, GC1_F_#1, GC1_F_OMe_#2, and GC1_F-MOE-OMe relatively suppressed PDGFC expression (Figure S4).

To further enhance the target gene effect of GC1-based nucleic acid *in vitro*, we optimized the nucleic acid modification. Based on GC1_F_#2 and GC1_OMe_#2, the former resulted in six types of nucleic acids (GC1_F_#2_d, GC1_F_#2_e, GC1_F_#2_f, GC1_F_#2_g, GC1_F_#2_h, and GC1_F_#2_i) and the latter in seven types (GC1_OMe_#2_d, GC1_OMe_#2_e, GC1_OMe_#2_f, GC1_OMe_#2_g, GC1_OMe_#2_h, GC1_OMe_#2_i, and GC1_OMe_#2_j) (Figure S5A). *In vitro*, GC1, GC1_F_#2_d, GC1_F_#2_g, GC1_OMe_#2_d, GC1_OMe_#2_g, and GC1_OMe_#2_h showed relative target gene suppression effects on LX2 activated by TGF-β (Figure S5B). We performed nucleic acid stability experiments for all nucleic acids using miR-29a-3. GC1, GC1_F_#2_d, and GC1_F_#2_g were highly stable (Figure S5C). Based on these results, we conducted *in vivo* experiments using GC1_F_#2_d and GC1_OMe_ #2_d.

Efficacy of the modified nucleic acid *in vivo*

In vivo evaluation of GC1_F_#2 _d and GC1_OMe_#2_d was performed using the CCl_4 model (Figure 2A). We compared tail vein and subcutaneous administration. Compared with miR-29a-3p, GC1_OMe_#2_d did not show a fibrosis-suppressing effect through either intravenous (i.v.) or subcutaneous (s.c.) administration. GC1_F_#2_d showed the same effect as miR-29a-3p when administered s.c., and tail vein administration showed a greater fibrosis-suppressing effect than miR-29a-3p administration (Figures 2B and 2C).

Next, the efficacy of nucleic acids was evaluated without atelocollagen. Using the mouse CCl_4 model, nucleic acids were turbidly mixed in physiological saline and administered through the tail vein (Figure S6A). Two dosage patterns were prepared for the modified nucleic acid: a normal dose (10 μg) and a double dose (20 μg). PSR staining was performed of the liver tissue after sacrifice (Figure S6B), and the efficacy of the nucleic acid was evaluated by comparing PSR-positive areas. The double dose of GC1_F_#2_d inhibited liver fibrosis in the absence of atelocollagen (Figure S6C).

Pharmacodynamics of oral administration of the modified nucleic acid

The pharmacodynamics of the orally administered nucleic acids (GC1_F_#2_d) were examined. After an 18-h fasting period, 10 μg modified nucleic acids labeled with Alexa Fluor 647 was orally administered, and the fluorescent label was monitored at 2, 4, and 24 h.

When the distribution of the labeled nucleic acid was observed using an *in vivo* imaging system (IVIS), it reached the small intestine after 2 h and was not detected in the intestinal tract after 24 h (Figure 3A). After 15, 30, and 60 min, oral administration of the labeled nucleic acid and its distribution in the head, heart and lungs, left and right kidney, liver, spleen and pancreas, small and large intestine, and testes and bladder were evaluated. Mice that did not receive labeled nucleic acids were used as controls (Figure 3B).
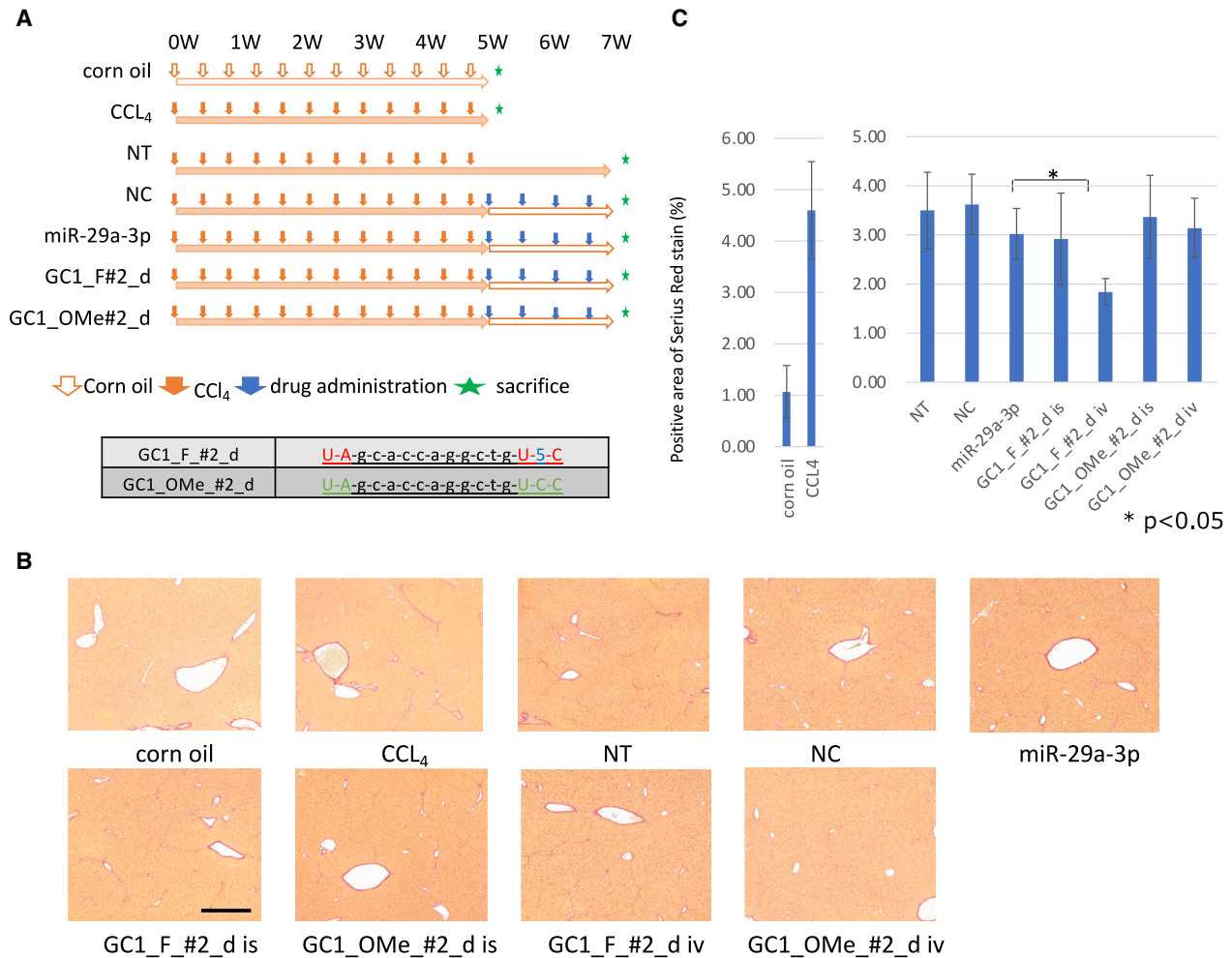
GC1_F_#2_d	U-A-g-c-a-c-c-a-g-g-c-t-g-U-5-C
GC1_OMe_#2_d	U-A-g-c-a-c-c-a-g-g-c-t-g-U-C-C

Figure 2. Target gene-inhibitory effect of the modified nucleic acid *in vivo*

(A) CCl_4 was intraperitoneally administered once every 3 days for 5 weeks (filled orange arrow), and corn oil was administered similarly as a control (unfilled orange arrow). The modified nucleic acid mixed with atelocollagen was administered through the tail vein once every 3 days (filled blue arrow). The green star indicates sacrifice. The experiment was conducted with 5 mice in each group. NT: CCl_4 administered for 5 weeks followed by no treatment. NC: after 5 weeks of CCl_4 treatment, a nucleic acid that does not recognize human genes was administered. miR-29a-3p: administered after 5 weeks of CCl_4 treatment. GC1_F_#2_d s.c./i.v.: after 5 weeks of CCl_4 treatment, GC1_F_#2_d was administered s.c. or i.v.. GC1_OMe_#2_d s.c./i.v.: After 5 weeks of CCl_4 treatment, GC1_OMe_#2_d was administered s.c. or i.v.. The structures of GC1_F#2_d and GC1_OMe#2_d are also shown. (B) PSR staining of the liver tissue. (C) Positive PSR staining areas of the liver tissue. Scale bar is indicated 200 μm. *$p < 0.05$, **$p < 0.01$. CCl_4, carbon tetrachloride; NC, negative control; NT, non-treatment.

The expression of Co1a1 in the liver was observed 4 and 24 h after oral administration of the modified nucleic acid compared with no treatment. In the control group, no significant difference was observed in the liver tissue after 24 h; however, there was a tendency to suppress target gene expression (Figure 3C).

The effects of orally administered nucleic acids were evaluated using a CCl_4 model. The group in which stimulation was performed with CCl_4 for the first 5 weeks, followed by oral administration of nucleic acids four times in 2 weeks (CCl_4_5w+NA_2w), was compared with the group in which physiological saline was administered four times in 2 weeks (CCl_4_5w+NT_2w). The PSR-posi-

tive area decreased in the nucleic acid administration group (Figures 4B and 4C). H&E staining showed no evidence of worsening of inflammation due to nucleic acid administration (Figure S6). Stimulation was performed with CCl_4 for 7 weeks, and in the final 2 weeks, the group in which nucleic acids were orally administered four times in 2 weeks (CCl_4_7w+NA_2w) was compared with the group in which physiological saline was administered four times in 2 weeks (CCl_4_7w+NT_2w). The PSR-positive area decreased in the nucleic acid administration group (Figures 4B and 4C), and no evidence of worsening inflammation due to nucleic acid administration was observed (Figure S7A). Regarding gene expression at the time of tissue collection, the
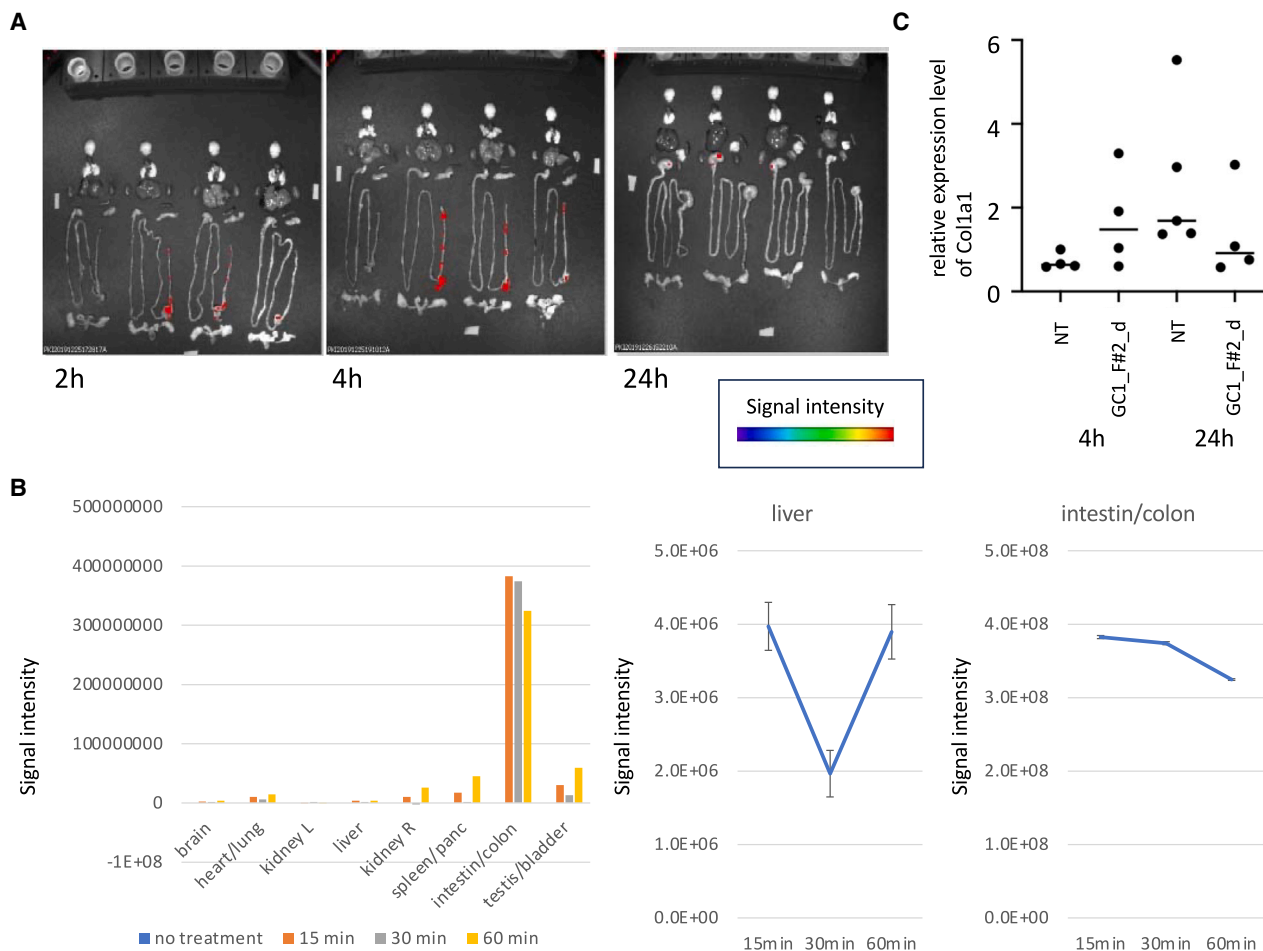
Figure 3. Body distribution with oral administration of the nucleic acid

(A) Nucleic acid distribution in the whole body 2, 4, and 24 h after oral administration of the nucleic acid labeled with Alexa 647. To ensure reproducibility, the nucleic acids were administered to four mice and analyzed in parallel. (B) Left: the presence of labeled nucleic acids in each organ and the signal intensity of Alexa 647 in each organ 15, 30, and 60 min after administration and without administration. Center and right: the abundance of nucleic acids in the liver and gastrointestinal tract 15, 30, and 60 min after administration. (C) Target gene suppression effect in the liver. Liver tissue was collected 4 and 24 h after administration of the nucleic acid; the experiment was conducted four times to compare the untreated and treated groups.

expression of both Col1a1 and Pdgfc tended to be suppressed in the nucleic acid group (Figure S7B).

DISCUSSION

Nucleic acid-based drug discovery for liver fibrosis that has been developed targets a single molecule, such as chaperone gp46,[28,29] COL1A1,[30] hydrogen peroxide-inducible clone 5,[31] and PDGFB.[32] However, liver fibrosis progresses in multiple steps.[33] Therefore, we investigated an miR-29a-based fibrosis control that targets both COL1A1 and PDGFC.[13] In this study, when nucleic acids were administered to a mouse model of chronic liver injury after CCl_4 stimulation, they improved liver fibrosis. Moreover, the progression of liver fibrosis was suppressed. Further, the degree and area of fibrosis staining decreased.

Nucleic acid drug discovery is the key to the commercialization of DDSs. The liver sinusoids are highly permeable[25] and have a high endocytic ability.[34] Since endocytosis is involved in the transport of substances from the liver sinusoids to the Disse cavity,[35] it is thought that nucleic acid transfer occurs from the liver sinusoids to hepatocytes. Therefore, the liver tissue is more advantageous for nucleic acid drug discovery than other organs. Furthermore, if the stability of the nucleic acid is increased, and its ability to bind to the target gene is improved, then it is expected that a smaller amount will be effective.

Modification of nucleic acids with 2'-OMe, 2'-MOE, 2'-F, LNA, etc. increases the structural stability of the nucleic acids, making them resistant to degradation by RNases in plasma and tissues, and is therefore expected to enable them to bind to target genes and exert their
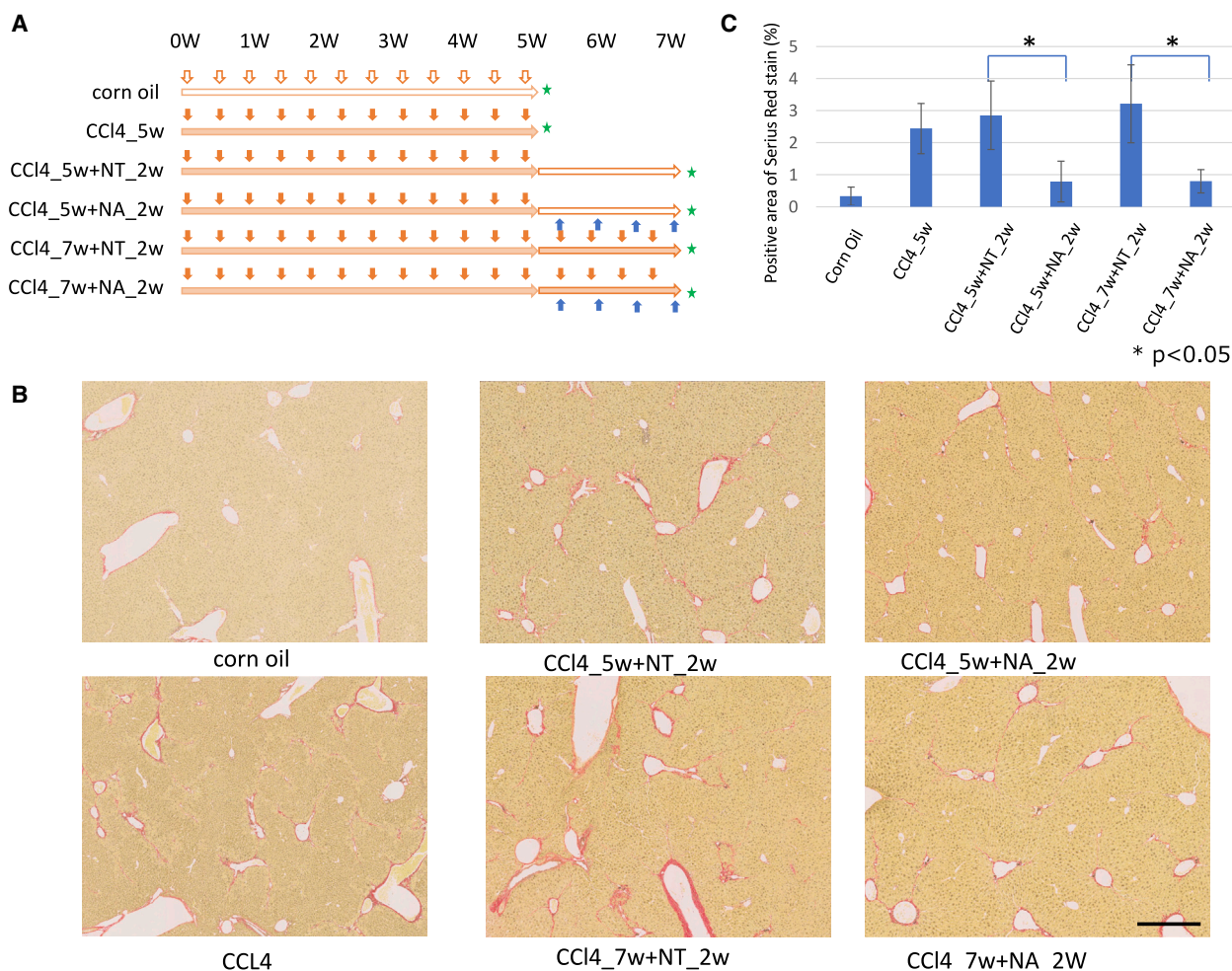
Figure 4. Anti-fibrotic effect with oral administration of the nucleic acid

(A) CCl_4 treatment and nucleic acid administration schedule. The analysis was performed in six groups (five mice each). The group received corn oil and CCl_4 for 5 weeks. The groups were as follows: CCl_4 for 5 weeks and then physiological saline (CCl_4_5w+NT_2w), nucleic acids (CCl_4_5w+NA_2w), CCl_4 for 7 weeks and then physiological saline for the last 2 weeks (CCl_4_7w+NT_2w), and nucleic acids (CCl_4_7w+NA_2w). (B) PSR staining of the liver tissue. (C) Comparison of PSR-positive staining areas. *$p < 0.05$.

functions.[36,37] The 2′-F modification is stable against nucleases and has the potential to extend its half-life in human plasma, increase nucleic acid binding affinity, and reduce immune stimulation.[38,39]

Nucleic acid target gene siRNAs are usually screened *in vitro*, and those that are effective are re-evaluated *in vivo*. However, owing to differences in gene transfer methods and *in vivo* drug dynamics, experimental results obtained *in vitro* do not necessarily correlate with *in vivo* experimental results. Therefore, based on *in vitro* experimental results, we verified nucleic acids with multiple structures using *in vivo* experiments as much as possible.

First, we optimized the siRNA structure, a short structure centered on the seed sequence, and the RNA-DNA hybrid structure. As expected, siRNA exhibited the highest target gene suppression effect, followed by the hybrid structure. The seed sequence structure had

a poor target gene suppression effect and was excluded from subsequent analyses (Figure S2). When we tested this *in vivo* using GC1 and siP-5, GC1 showed more fibrillar siRNA than siP-5 (S Figure 3). Therefore, we decided to proceed with the analysis by focusing on GC1. Next, we evaluated the effect of the modification group on suppression of the target gene *in vitro* and found that modification with the F and OMe groups had a relatively good effect on suppressing the target gene (Figure S4). We further optimized the modification group and evaluated the stability of nucleic acids with S7 nuclease and found that the F group modification was more stable than the OMe group modification (Figure S5). Two species, GC1_F_#2_d and GC1_OMe_#2_d, were used for *in vivo* experiments.

In the *in vivo* experiment, two administration methods were selected: systemic (i.v.) and local (s.c.). Systemic administration had

a greater fibrosis-inhibiting effect than local administration, and GC1_F_#2_d had a greater fibrosis-inhibiting effect than miR-29a-3p (Figure 2). When examining whether nuclease-resistant nucleic acids were effective without a DDS, we found that systemic administration without atelocollagen had a dose-dependent inhibitory effect on liver fibrosis (Figure 3). Dosing experiments were then conducted.

Whole-body imaging analysis of labeled nucleic acids showed that the nucleic acids remained in the gastrointestinal tract for 2–24 h. Next, the amount of labeled nucleic acid present in each organ was evaluated; the nucleic acid was present in the gastrointestinal tract 15 min after administration and in the liver. In addition, the target gene suppression effect in the liver was not observed 4 h after administration but was suppressed 24 h later (Figure 4).

HSCs exist as cytokine-producing HSCs in the resting phase, and when activated by TGF-β, they change to myofibroblastic HSCs and produce type I collagen, which is used by pre-neoplastic hepatocytes. Transcriptional coactivator with PDZ-binding motif (TAZ) activation is associated with increased stiffness and with hepatocarcinogenesis. Furthermore, when hepatitis inflammation improves, activated HSCs may return to resting HSCs.[40] Administration of miR-29a-3p may suppress fibrosis and carcinogenesis owing to its COL1A1 suppressing effect.

In conclusion, we developed a microRNA-based nucleic acid that avoids degradation by ribonucleases and stably expresses its function in target organs. Although microRNAs generally have lower target gene expression levels than siRNAs, they can control many target genes simultaneously. The administration of nucleic acids that are difficult to degrade in vivo does not cause toxicity in target organs. This nucleic acid can control multiple target genes and is a promising oral drug that can directly control liver fibrosis.

MATERIALS AND METHODS

Cell culture and nucleic acid transfection
The human HSC cell line LX2, donated by Scott L. Friedman (Division of Liver Diseases, Mount Sinai Hospital) was cultured using Dulbecco's modified Eagle's medium (Wako Pure Chemicals Industries, Osaka, Japan) and supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Thermo Fisher Scientific, MA, USA), and nonessential amino acids (Thermo Fisher Scientific).[41] 1×10^5 LX2 cells were seeded in 6-well plates (Thermo Fisher Scientific), and 40 μM nucleic acid was transfected using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). All modified nucleic acids were synthesized and purchased from Bio-Pharma (Osaka, Japan). For the negative control, mirVana miRNA Mimic, Negative Control #1, was used (Thermo Fisher Scientific).

In the experimental system of LX2 cells with and without TGF-β treatment, TGF-β (2.5 ng/mL; Sigma-Aldrich, MO, USA) was administered on day −1, nucleic acid transfection was performed on day 0, and cells were harvested on day 1 or 2.

RNA extraction and mRNA and microRNA expression analyses
The total RNA from liver cells was extracted using the miRNAeasy Mini Kit (QIAGEN, Venlo, the Netherlands), according to the manufacturer's instructions. Complementary DNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). FastStart Universal SYBR Green Master Mix (Roche) was used for real-time PCR analysis. The primer sequences are listed in Table S1. The reactions were performed in triplicate. A StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) was used to detect microRNAs and mRNA. The reactions were performed in triplicate.

Mouse model of liver fibrosis
Carbon tetrachloride (CCl_4)-treated mice were used as a model of chronic liver injury. The experimental procedure has been described previously.[13] Six-week-old male C57BL/6J mice (Japan SLC, Shizuoka, Japan) were acclimated for 1 week, and lighting conditions were controlled with a 12-h light-dark cycle. The animal protocol was approved by the institutional animal care and use committee of Tokyo Medical University (permission R3-0091).

Administration of nucleic acid in vivo
Fifty micrograms of nucleic acid was mixed with 200 μL of atelocollagen for systemic administration (AteloGene Systemic Use; Koken, Tokyo, Japan) and injected into the tail vein every 3 days. In addition, atelocollagen (AteloGene Local Use) was injected s.c. every 3 days for local administration (Figures 2 and S2). For the group not administered atelocollagen, 50 and 100 μg nucleic acid were mixed with phosphate-buffered saline and administered via the tail vein, respectively (Figure S6). For the group not administered a DDS, 10 μg nucleic acid mixed with 500 μL saline was administered via a stomach tube (Figure 4).

Pharmacodynamic study
Twenty micrograms of Alexa Fluor 647-labeled nucleic acid mixed with 500 μL saline was administered via a stomach tube (Figure 3). After 2, 4, and 24 h, the mice were euthanized with isoflurane, and Alexa Fluor 647 signals were tracked throughout the body using IVIS (Caliper Life Sciences, Hopkinton, MA, USA). Furthermore, 15, 30, and 60 min after administration of the labeled nucleic acid, its distribution was observed throughout the body. The intensity of Alexa 645 in the brain, heart, lungs, kidneys, spleen, liver, pancreas, intestine, colon, testes, and bladder of mice was measured, and Alexa 645 fluorescence color images and background photon images were superimposed using Living Image v.1.11 software overlay and IGOR image analysis software (v.4.02 A; Wave Metrics, Lake Oswego, OR, USA).

Histological analysis
Liver specimens were fixed with 10% formalin, and two 5-μm serial paraffin sections were made. One section was stained with H&E and the other with PSR (Sigma-Aldrich) and counterstained with Fast Green FCF (Sigma-Aldrich).[42] PSR-positive areas were quantified using a microanalyzer (Nihon Poladigital, Tokyo, Japan). To

validate these areas, five images containing at least one portal vein were chosen.

RNA stability assay

One microgram of nucleic acid was incubated at 37°C in 50 μL of 50 mM Tris-HCl (pH 8), containing 5 mM $CaCl_2$ and 0.5 units S7 nuclease (Roche Diagnostics, Japan). After 10 min, the S7 nuclease reaction was stopped by addition of 5mM EDTA, and the samples were thawed and run on a 7 M urea-15% polyacrylamide gel. The gel was stained with SYBR Green II and analyzed using E-BOX-VX2 (M&S Instruments, Japan).

Statistical analysis

Analysis of variance and Tukey's honest significant difference post hoc tests were used for multiple-group data, and Student's t test was used for independent two-group data (Statistical Package for the Social Sciences, IBM, Chicago, IL). Statistical significance was set at $p < 0.05$.

DATA AND CODE AVAILABILITY

All data are included in the paper or the supplemental information. Additional data are available from the lead contact upon reasonable request.

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AUTHOR CONTRIBUTIONS

Study concept and design, Y. Murakami; acquisition of data, T.U., M.T., K.F., A.I., Y.H., and Y. Matsumoto; statistical analysis, T.U. and Y. Matsumoto; interpretation of data, T.U., M.T., K.F., Y.H., Y. Matsumoto, and Y. Murakami; drafting of the manuscript, T.U., M.T., Y. Matsumoto, and Y. Murakami; critical revisions of the manuscript for important intellectual content, M.K.; funding and administrative support, Y. Murakami.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2024.102438.

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